Handbook of Experimental Pharmacology 192

Jack E. Henningfield
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Nicotine Psychopharmacology

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Volume 192

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Preface

The fact that tobacco ingestion can affect how people feel and think has been known for millennia, placing the plant among those used spiritually, honorifically, and habitually (Corti 1931; Wilbert 1987). However, the conclusion that nicotine accounted for many of these psychopharmacological effects did not emerge until the nineteenth century (Langley 1905). This was elegantly described by Lewin in 1931 as follows: "The decisive factor in the effects of tobacco, desired or undesired, is nicotine. . . " (Lewin 1998). The use of nicotine as a pharmacological probe to understand physiological functioning at the dawn of the twentieth century was a landmark in the birth of modern neuropharmacology (Limbird 2004; Halliwell 2007), and led the pioneering researcher John Langley to conclude that there must exist some "receptive substance" to explain the diverse actions of various substances, including nicotine, when applied to muscle tissue (Langley 1905).

Research on tobacco and nicotine progressed throughout the twentieth century, but much of this was from a general pharmacological and toxicological rather than a psychopharmacological perspective (Larson et al. 1961). There was some attention to the effects related to addiction, such as euphoria (Johnston 1941), tolerance (Lewin 1931), and withdrawal (Finnegan et al. 1945), but outside of research supported by the tobacco industry, addiction and psychopharmacology were not major foci for research (Slade et al. 1995; Hurt and Robertson 1998; Henningfield et al. 2006; Henningfield and Hartel 1999; Larson et al. 1961). This situation changed rapidly in the 1970s and 1980s with a virtual explosion of research focused on nicotine psychopharmacology and potential addictive effects (US DHHS 1979, 1988; National Institute on Drug Abuse 1984, 1987 (Henningfield and Goldberg 1983).

The expansion of nicotine-related research was driven largely by the growing recognition of the emerging tobacco epidemic. It was facilitated by advances in research methodology and technology that enabled scientists to examine the cellular and even molecular basis of nicotine action. Such developments contributed to a rapidly increasing understanding of the effects of nicotine on brain structure and

function, as well as to identifying and characterizing the effects of the multitude of subtypes of nicotinic receptors, laying the foundation for advances that might lead to therapeutic uses of nicotine and related molecules beyond their use for the treatment of tobacco dependence and withdrawal (Henningfield et al. 2006; Buchhalter et al. 2008).

An update on the remarkable progress in research related to nicotine psychopharmacology was presented in a special issue of the journal *Psychopharmacology* in 2006. The volume clearly struck a chord with many in basic science, public health, and policy, who learned that this area of pharmacological science was not only strong, but also highly relevant to potential public health policy and regulatory efforts aimed at controlling tobacco use, addiction, and resultant deadly disease. This was anticipated in the mid 1990s when the Commissioner of the United States Food and Drug Administration (FDA) proposed that the agency regulate tobacco products (Kessler 2001; Kessler et al. 1997; FDA 1995, 1996). The Commissioner's testimony and recommendations were based in part on basic science findings, including the actions of nicotine on nicotinic receptors in the brain, advances in understanding the mechanisms of action of nicotine through neuroimaging, and discriminative and reinforcing actions of nicotine. Subsequently, the World Health Organization came to rely in part on psychopharmacological research findings as part of the science base for development and implementation of its international treaty, proposed in the late 1990s, which entered into force in 2005 (WHO 2005). The Treaty's articles that include attention to nicotine dosing capacity and effect, in particular, will continue to rely on psychopharmacology research as they are implemented.

The European Commission has also taken a strong science-based approach to tobacco disease control and product regulation and has made tobacco control a priority since the mid 1980s. For examples, reports by the Analysis of Science Policy in Europe for Control of Tobacco (ASPECT) Consortium financed by and prepared for the use of the European Commision, Directorate-General for Health and Consumer Protection emphasize the need for a strong science base for tobacco-control policy and interventions (European Commission 2004, 2007). There are many other national and regional efforts as well, but these illustrate the global public health and regulatory importance of nicotine and tobacco science that has included psychopharmacological research.

The fact that psychopharmacological research on nicotine and related compounds was progressing at a rapid pace, with broad and substantial interest, indicated that an update, in the form of a systematically planned and edited special volume, could serve the field and facilitate scientific progress. It was challenging to represent the many promising areas of research, from molecular to clinical to epidemiological, within a single volume. We asked leading researchers to write relatively focused reviews on their areas of recent interest. Each article was reviewed by experts, including other authors whose articles are published in this volume, producing what we believe is a reference that will be useful to researchers, students, health professionals, and to the growing number of people involved in efforts to regulate tobacco product contents and designs nationally and internationally. This work was intended

as a contribution to the reversal of the current tobacco epidemic and thereby to preventing many of the approximately one-half billion tobacco attributable deaths predicted in the first half of the twentieth century (Koop 2004; Doll 1994).

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Part I
Nicotine and Tobacco Consumption: Measurement and Trends

Global Patterns of Nicotine and Tobacco Consumption

S. Katharine Hammond

Contents

Abstract Humans consume tobacco in dozens of guises, all of which are toxic; globally, a tenth of deaths among adults are caused by tobacco. Tobacco may be combusted (e.g., cigarettes, bidis, kreteks); heated (e.g., waterpipes, hookah, nargile); or taken orally or nasally (e.g., snuff, betel quid, chewing tobacco). The predominant forms vary among cultures, but the use of cigarettes has grown most dramatically in the past century. While smoking rates among women are comparable to those among men in Europe and North America, in other regions the rate is ten or more times higher among men; this gender gap is closing among young people. Per capita tobacco use in the USA doubled in the first half of the twentieth century, and has since declined to less than the 1900 levels. While cigarettes were only 2% of tobacco consumed in the USA in 1900 (half was chewing tobacco) 50 years later they were over 80%. A similar increase in tobacco consumption, and a shift to cigarettes, has been occurring globally, with a concomitant increase in tobacco-related death and disease that is not expected to peak for another two decades.

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1 A History of Tobacco

Tobacco is a word bombarding people daily in cigarette advertisements and from rows of tobacco products in stores, all of which leads them to feel they have a clear definition. The word *tobacco* was in fact a name applied in error to the plant that European explorers witnessed smoked by Native Americans. The name originally referred instead to the cane pipe, called a *tabaco* or *tavaco,* used to sniff smoke (Charlton 2004). The plant has been called by many names: in Mexico it was called *yetl*, *picietl* or *piciete*; in Mayan regions it was *kutz*; and it was known as *sayri* in Peru (Charlton 2004). As tobacco spread to Europe and its use evolved, the nomenclature expanded: *petun*, *kohaba*, *uppowoc*, *Tsâla*, *o-yen'-kwa*, *herbe sainte*, *l'herbe du Grand Prieur*, *l'herbe medicée*, *killikinnick*, and *American silver weed*; all are names for one of humanity's most enduring sidekicks (Kell 1966). Of the sixty species of *Nicotiana*, most are indigenous to America (Charlton 2004). Smoked, mixed with lime and chewed, snuffed, drunk, and used for enemas, tobacco has been a constant companion in America for thousands of years. Mayans in Central America burned and inhaled the smoke from tobacco 2500 years ago in religious settings (Doll 1999). In Mexico, the Aztecs mixed tobacco with the charred remains of poisonous animals, one seed of *oloiuhqui* and hairy black worms to form an ointment called *Teotlacualli* (Food of the God) for divine consumption (Elferink 1983). Tobacco was considered to possess both divine puissance and healing abilities by peoples throughout the Americas. Walter Raleigh and his contemporaries introduced tobacco into English society, while the Spanish and Portuguese explorers of the fifteenth and sixteenth centuries are credited with its first movement east—to Europe and beyond. However, recent archaeological evidence could suggest otherwise.

In 1993, Franz Parchse, Svetlana Balabanova, and Wolfgang Pirsig published their findings of nicotine concentrations in Peruvian mummies dating from 200–1500 AD but also found unexpected concentrations in Egyptians mummies (1070 BC–395 BC), in skeletal tissue from Sudan (5000–4000 BC and 400–1400 AD), as well as in remains from South Germany's Bell Culture (c. 2500 BC) (Parsche et al. 1993). These traces of nicotine have been the subject of a great amount of debate as to their origin. When Balabanova, Rosing, Buhler, Hauser and Rosenthal discovered reasonably high nicotine concentrations in eighteen individuals from the *Reihengräberfeld* (graveyard) of Kirchheim unter Teck (Baden Württemberg, Germany) dated from 450–700 AD, they postulated that *Nicotiana* was known in Central Europe before the Spanish and Portuguese expeditions to America (Balabanova et al. 2001). Those eighteen individuals had nicotine levels ranging from 32 to 150 $\mathrm{ng\,g^{-1}}$ (Balabanova et al. 2001). Similarly; Hirat Behari Routh found documents indicating tobacco use by not only South and Central American indigenous populations but also Egyptian, Persian, Chinese and African populations (Routh et al. 1998).

Despite these discoveries and the growing scholarship in ancient tobacco use throughout the world, the most clearly documented use of tobacco before European exploration of the Western Hemisphere comes from South and Central America. Amerigo Vespucci recorded Indians on the Island of Margarita chewing the green

leaves of an herb mixed with lime as a thirst quencher in 1499, and in 1500, Alonso Niño and Cristobal Guerra recorded the same practice with a slightly different twist: the lime and tobacco mixture was used to whiten teeth (Stewart 1967). However, tobacco was purported to cause hallucinogenic effects as well: Spanish chroniclers describe the effects of tobacco as drunkenness and loss of senses and they depicted populations in Mexico using tobacco to ward off animals (Elferink 1983). When Durán portrayed the preparation of *Teotlacualli*, he described priests smearing the ointment on their bodies and becoming wild as if a wholly different and slightly crazed person (Elferink 1983). The hallucinogenic properties of tobacco are a common theme in Spanish chronicles, as well as its property as both a stimulant and sedative.

Though evidence suggests possible nicotine consumption before Columbus' trip to America, his voyage and the explorations of his contemporaries and successors popularized tobacco in European society. In the mid-sixteenth century Jean Nicot, the French ambassador to Lisbon for whom nicotine was named, promoted the great healing powers of tobacco, which he claimed to cure everything from headaches to syphilis (Charlton 2004). In 1587, Giles Everard published his work in Antwerp describing tobacco as a panacea and nepenthe, which became a popular view of doctors throughout Europe who hailed the many curative properties of the plant (Harley 1993). Tobacco became widely popular with the English aristocracy when Walter Raleigh, a favorite of Elizabeth I, brought it to England after returning from one of his expeditions to the New World. While the Tudors took up tobacco as a sign of their colonial ambitions in America, due to its associations with Raleigh (who was not greatly liked by the new court of James I, imprisoned more than once in the Tower of London; and executed by James in 1618), tobacco also became associated with atheism and everything wrong with society in early-modern England (Harley 1993). During this time (shortly after its introduction in England), the largely Puritan population at Cambridge publicly criticized tobacco (Harley 1993). James I of England wrote a short work on the evils he saw in the plant in 1604 called "A Counterblaste to Tobacco" in which he writes, "Yet it makes a kitchin also oftentimes in the inward parts of men, soiling and infecting them, with an unctuous and oily kind of soote, as hath bene found in some great *Tobacco* takers, that after their death were opened" (James 1604). King James I, unlike his predecessor Elizabeth, decried the use of tobacco as vain and rejected the opinions of doctors who told of its healing capabilities, but he was not the only person to do so. Criticisms of the new plant and reports of its ill effects counterpoised tales of miraculous healing from the time of its introduction into Europe.

Despite the criticisms levied against tobacco use by James as well as doctors across Europe in opposition to the idea of the great catholicon from the New World, tobacco spread through Europe in the early seventeenth century and beyond. Pipe smoking spread from England to the Netherlands in the early seventeenth century where it can be seen depicted in the period's great works of art (Doll 1999). Pipe smoking made its way to Egypt in 1601–1603 and to Turkey in 1605 (Robinson 1985). As tobacco traveled through Europe and Asia, the method of use changed as well. During the seventeenth century snuff was the most popular

use of tobacco; by the eighteenth century cigars were the rage, and at the end of the nineteenth century cigarettes had made their way to the top of the list due to their affordability and aesthetic appeal (Doll 1999).

By 1600, the plant had spread through Europe to Italy, Spain, France, England, Belgium, and Switzerland and had begun its move beyond Europe to Japan, China, Indian, Java, Africa, and the Philippines (Mancall 2004). Tobacco had reached East Asia three-quarters of a century after Columbus' voyage to the Americas: it made its way to the Philippines in 1575; to Japan in 1590; Macao in 1600; Java by 1601; Ceylon in 1610; Korea by 1616; and was present in China by the first quarter of the seventeenth century (Goodrich 1938). Records of its use and the immediate appeal of the plant are widespread. Tobacco, commonly smoked in pipes, was first banned in China shortly after its introduction: in 1637 (Goodrich 1938). Jacob le Maire first records the smoking of tobacco in 1616 in New Guinea (Laufer 1931). In India the first major tobacco crops predate 1620 and were introduced by Portuguese sailors (Gokhale 1974). There, the tobacco industry also helped to stimulate the metalwork and pottery industry for the production of *hookahs* and *chilime* ("a short pipe with a wide opening, a tapering cylindrical body with a narrow mouthpiece which was covered with a cloth while smoking"), which were the most popular methods of use in the area (Gokhale 1974). Tobacco was most far-reaching in Africa where it became incorporated into creation myths (Mancall 2004). In Cameroon, production of tobacco pipes is a skilled and intricate art practiced for hundreds of years, while the South African Bantu traditionally use snuff for ceremonial purposes (Gebauer 1972).

2 Tobacco Today

Tobacco is a leading cause of death globally, responsible for one in ten deaths of among adults (5 million people annually), and the toll is expected to double over the next two decades; 70% of these deaths will be in developing countries. Today, every 6 s someone dies from tobacco caused disease. While tobacco use is declining in developed countries, it is increasing dramatically in developing countries. Furthermore, the taboos that have protected women from tobacco are falling, so that increases in the proportion of women who smoke or use smokeless tobacco may dramatically affect disease rates in the future beyond most predictions. For instance, while the smoking rate among men in South East Asia is ten times that for women there, the rate among 13–15-year-old boys is two and half that for girls.

2.1 The Gender Gap

Figures 1 and 2 present the smoking rates among men and women globally (details are in Table 1). Many observations can be made from these data.

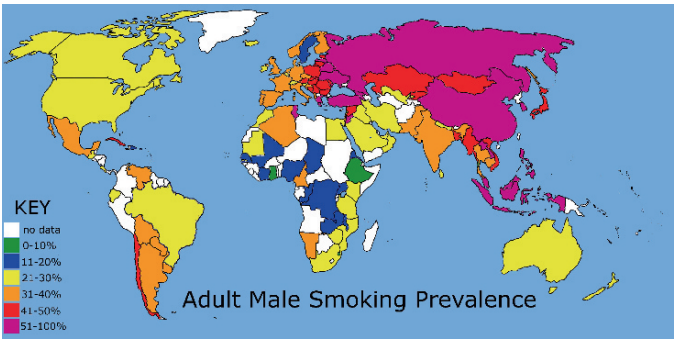

Fig. 1 Adult male smoking prevalence (constructed from data in MPOWER; World Health Organization 2008)

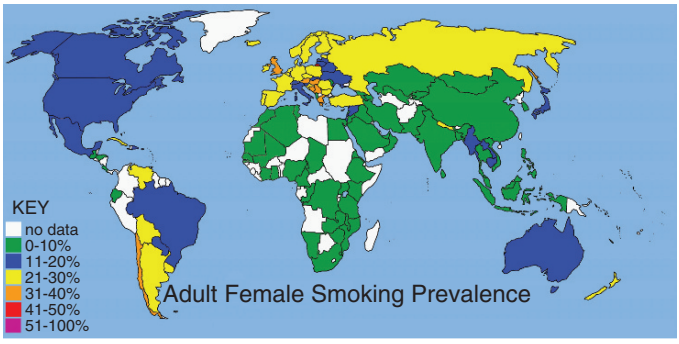

Fig. 2 Adult female smoking prevalence. (constructed from data in MPOWER; World Health Organization 2008)

First, the smoking rates among men are quite high and exceed 30% prevalence in much of the world; each continent has at least one country where over a fourth of men smoke (Fig. 1). The highest rates are seen in the Western Pacific Region, South-East Asia, and Eastern Europe, including the Russian Federation. In all four of the most populous nations, more than a quarter of adult men smoke, and these rates exceed 60% among men in China and Indonesia. While 31% of men in India smoke tobacco, over half of them use tobacco products, which reflects the high rate of oral tobacco use there. The smoking rate in the USA has been declining for four decades, from 52% of men in 1965 (Centers for Disease Control and Prevention 1994) to 24% in 2006 (Rock et al. 2007).

The smoking rates among adult women present a very different picture (Fig. 2). Less than 10% of women smoke in Africa and much of South-East Asia and China, and in only a few countries do more than 20% of women smoke (Russian Federation, most of Central and Western Europe, Turkey, New Zealand, Argentina, Venezula, and a few other Latin American countries). In general, smoking rates among women are closer to those among men in developed countries (42% men,

Table 1 Adjusted prevalence estimates for WHO Member States

Country	Smoking any tobacco product [%][a]				Smoking cigarettes [%][b]			
	Males		Females		Males		Females	
	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]
Africa								
Algeria	31.3	29.5	0.3	0.2	28.7	27.0	0.2	0.2
Angola	...	...	...	...	...	...	...	...
Benin	...	...	...	...	...	...	...	...
Botswana	...	...	...	...	...	...	...	...
Burkina Faso	20.3	16.3	8.2	7.6	15.5	11.2	0.9	0.3
Burundi	...	...	...	...	...	...	...	...
Cameroon	12.9	10.6	2.7	2.0	10.1	7.6	1.8	1.3
Cape Verde	...	...	...	...	...	...	...	...
Central African Republic	...	...	...	...	...	...	...	...
Chad	14.0	10.9	2.2	1.5	11.2	8.0	1.1	0.7
Comoros	23.1	17.8	10.4	8.6	20.3	15.1	4.0	2.7
Congo	12.3	8.9	0.8	0.5	9.9	6.6	0.4	0.2
Cote d'Ivoire	15.4	11.4	1.7	1.1	12.5	8.7	0.6	0.2
Democratic Republic of the Congo	13.8	10.4	1.6	1.2	11.2	7.7	0.4	0.3
Equatorial Guinea	...	...	...	...	...	...	...	...
Eritrea	16.1	11.6	1.1	0.6	15.2	10.8	0.7	0.3
Ethiopia	5.8	3.8	0.6	0.3	5.3	3.5	0.4	0.2
Gabon	...	...	...	...	...	...	...	...
Gambia	27.8	25.5	2.3	1.8	18.0	14.4	0.5	0.2
Ghana	8.2	5.9	0.7	0.3	6.0	3.8	0.5	0.2

	Guinea	...	...	...	...	...	...	...	...
	Guinea-Bissau	...	...	...	...	...	...	...	...
	Kenya	24.1	18.6	1.6	0.9	22.2	16.7	0.9	0.4
	Lesotho	...	...	...	...	...	...	...	...
	Liberia	...	...	...	...	...	...	...	...
	Madagascar	...	...	...	...	...	...	...	...
	Malawi	19.3	14.8	4.0	3.3	16.6	12.3	1.6	1.0
	Mali	19.2	15.1	2.5	1.7	15.3	11.1	0.8	0.3
	Mauritania	23.1	19.2	3.7	2.8	17.9	13.7	0.8	0.3
	Mauritius	36.2	29.1	1.1	0.6	36.2	29.1	1.1	0.6
	Mozambique	21.4	15.8	3.2	2.0	20.0	14.6	1.5	0.8
	Namibia	31.0	24.8	9.3	7.5	28.9	22.6	7.9	5.9
	Niger	...	...	...	...	...	...	...	...
	Nigeria	11.7	8.6	0.9	0.6	9.0	6.0	0.2	0.1
	Rwanda	...	...	...	...	...	...	...	...
	Sao Tome and Principe	22.0	21.3	10.6	8.6	22.0	21.3	10.6	8.6
	Senegal	19.9	15.8	1.3	0.8	15.7	11.5	0.6	0.2
!	Seychelles	35.5	28.4	7.0	5.1	31.5	24.4	3.0	1.8
	Sierra Leone	...	...	...	...	...	...	...	...
	South Africa	27.1	20.9	8.9	6.7	24.9	18.8	7.6	5.3
	Swaziland	11.9	7.8	2.1	1.3	11.0	7.1	1.9	1.0

(continued)

Table 1 (continued)

Country		Smoking any tobacco product [%][a]				Smoking cigarettes [%][b]			
		Males		Females		Males		Females	
		Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]
	Togo	...	...	...	...	...	...	...	...
	Uganda	17.3	13.2	2.5	1.6	15.7	11.7	1.2	0.6
	United Republic of Tanzania	21.2	16.1	3.3	2.4	18.8	13.8	1.4	0.8
!	Zambia	17.0	12.8	3.5	2.5	15.0	10.8	1.5	0.9
	Zimbabwe	20.8	15.8	2.9	2.0	18.4	13.4	1.3	0.8
The Americas									
	Antigua and Barbuda	...	...	...	...	...	...	...	...
	Argentina	34.6	27.0	24.6	21.1	34.3	26.1	22.7	18.1
	Bahamas	...	...	...	...	...	...	...	...
!	Barbados	18.5	16.6	3.3	2.7	17.1	15.2	2.5	2.0
	Belize	...	...	...	...	...	...	...	...
	Bolivia	35.8	32.6	29.8	27.0	35.7	32.1	27.3	24.1
*	Brazil	...	16.8	...	9.5	...	16.3	...	8.4
!	Canada	...	...	...	...	...	...	...	...
!	Chile	42.6	40.1	33.3	32.8	42.2	39.4	30.1	29.1
	Colombia	...	...	...	...	...	...	...	...
	Costa Rica	26.7	10.0	7.3	2.5	26.7	10.0	7.3	2.5
	Cuba	44.8	44.4	29.6	26.1	37.0	36.5	27.3	24.0
	Dominica	...	...	...	...	...	...	...	...
	Dominican Republic	14.9	13.1	11.0	9.4	13.6	11.8	9.4	8.0
	Ecuador	23.9	5.8	5.4	1.3	23.6	5.5	5.2	1.2

El Salvador	...	...	...	...	...	...	...	...
Grenada	...	...	...	...	...	...	...	...
Guatemala	24.8	7.8	3.9	0.8	24.8	7.8	3.9	0.8
Guyana	...	...	...	...	...	...	...	...
Haiti	...	...	...	...	...	...	...	...
Honduras	...	...	3.3	0.6	...	...	3.3	0.6
Jamaica	19.6	17.9	8.9	7.5	17.7	15.9	7.5	6.3
Mexico	37.6	22.1	12.4	6.1	37.6	22.1	12.4	6.1
Nicaragua	...	...	...	...	...	...	...	...
Panama	...	...	...	...	...	...	...	...
Paraguay	33.4	22.1	14.9	6.7	33.2	21.3	14.4	5.8
Peru	...	...	...	...	...	...	...	...
Saint Kitts and Nevis	...	...	...	...	...	...	...	...
Saint Lucia	28.0	26.9	11.0	9.5	25.0	23.6	8.2	7.0
Saint Vincent and the Grenadines	17.6	16.0	5.2	4.4	17.6	16.0	5.2	4.4
Suriname	...	...	...	...	...	...	...	...
Trinidad and Tobago	36.5	36.3	7.3	6.2	32.6	32.3	5.6	4.7
United States	25.7	20.5	20.3	15.8	25.7	20.5	20.3	15.8
Uruguay	36.6	34.3	25.8	24.3	36.6	34.3	25.8	24.3
Venezuela	33.4	26.0	27.8	25.1	33.4	26.0	27.8	25.1

(continued)

Table 1 (continued)

Country	Smoking any tobacco product [%][a]				Smoking cigarettes [%][b]			
	Males		Females		Males		Females	
	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]
Eastern Mediterranean								
Afghanistan	...	...	...	...	...	...	...	...
Bahrain	26.2	13.4	2.7	1.4	25.7	12.0	2.1	1.0
Djibouti	...	...	...	...	...	...	...	...
Egypt	26.0	24.4	4.1	4.1	22.7	21.1	3.5	3.5
Iran (Islamic Republic of)	26.2	20.2	4.5	3.2	21.4	15.8	1.7	1.0
Iraq	25.7	9.9	1.9	1.0	25.2	8.8	1.3	0.6
Jordan	61.7	61.7	7.9	7.9	61.4	61.4	7.9	7.9
Kuwait	...	...	...	...	...	...	...	...
Lebanon	29.0	27.3	6.9	6.9	29.0	27.3	6.9	6.9
Libyan Arab Jamahiriya	...	...	...	...	...	...	...	...
Morocco	29.5	27.9	0.3	0.3	26.8	25.2	0.3	0.2
Oman	24.8	11.4	1.0	0.2	24.4	10.3	0.3	0.0
Pakistan	31.7	25.3	5.2	3.9	26.7	20.3	2.2	1.4
Qatar	...	...	...	...	...	...	...	...
! Saudi Arabia	25.6	12.9	3.2	1.9	25.2	11.8	3.0	1.7
Somalia	...	...	...	...	...	...	...	...
Sudan	...	...	...	...	...	...	...	...
Syrian Arab Republic	42.0	38.6	...	...	41.2	37.8	...	...
Tunisia	51.0	49.2	1.7	1.6	47.4	45.5	1.0	0.9
! United Arab Emirates	27.2	16.0	2.4	1.1	26.8	14.9	1.7	0.5
West Bank and Gaza Strip	...	...	...	...	...	...	...	...
Yemen	...	...	...	...	...	...	...	...

Europe								
Albania	39.6	36.5	3.9	2.6	39.6	36.5	3.9	2.6
Andorra	35.7	32.2	24.5	20.6	35.7	32.2	24.5	20.6
Armenia	52.9	47.0	4.0	2.8	52.9	47.0	4.0	2.8
Austria	45.5	39.9	35.8	35.8	45.5	39.9	35.8	35.8
Azerbaijan	. . .	. . .	0.9	0.4	. . .	. . .	0.9	0.4
Belarus	63.6	57.6	17.4	13.8	63.6	57.6	17.4	13.8
Belgium	28.8	22.0	21.5	18.3	28.8	22.0	21.5	18.3
Bosnia and Herzegovina	48.8	45.1	32.0	28.7	48.8	45.1	32.0	28.7
Bulgaria	44.6	38.8	21.8	18.3	44.6	38.8	21.8	18.3
Croatia	37.5	33.8	25.4	22.0	37.5	33.8	25.4	22.0
Cyprus	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
Czech Republic	35.9	29.7	23.4	19.3	35.9	29.7	23.4	19.3
Denmark	35.8	28.8	29.4	24.2	35.8	28.8	29.4	24.2
Estonia	49.0	41.3	25.3	19.7	49.0	41.3	25.3	19.7
Finland	30.7	24.0	21.0	15.4	30.7	24.0	21.0	15.4
France	34.4	28.3	22.7	20.1	34.4	28.3	22.7	20.1
Georgia	55.8	49.7	5.8	3.8	55.8	49.7	5.8	3.8
Germany	36.0	29.5	22.0	19.2	36.0	29.5	22.0	19.2
Greece	62.4	59.4	32.8	29.0	62.4	59.4	32.8	29.0
Hungary	44.6	38.2	30.5	27.0	44.6	38.2	30.5	27.0
Iceland	25.7	19.2	25.2	18.9	25.7	19.2	25.2	18.9
Ireland	25.0	18.6	23.8	17.5	25.0	18.6	23.8	17.5

(continued)

Table 1 (continued)

Country		Smoking any tobacco product [%][a]				Smoking cigarettes [%][b]			
		Males		Females		Males		Females	
		Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]
Israel	30.9	27.3	17.6	14.8	30.9	27.3	17.6	14.8	
Italy	30.6	27.0	16.4	13.2	30.6	27.0	16.4	13.2	
Kazakhstan	43.9	37.0	9.8	6.5	43.9	37.0	9.8	6.5	
Kyrgyzstan	45.0	38.6	2.2	1.2	45.0	38.6	2.2	1.2	
Latvia	53.2	45.9	19.1	13.9	53.2	45.9	19.1	13.9	
Lithuania	44.4	36.7	17.6	11.9	44.4	36.7	17.6	11.9	
Luxembourg	37.1	31.3	27.3	25.3	37.1	31.3	27.3	25.3	
Malta	32.0	28.5	21.8	18.1	32.0	28.5	21.8	18.1	
Monaco	...	...	...	...	...	...	...	...	
Montenegro	...	...	...	...	...	...	...	...	
Netherlands	38.3	31.5	28.5	26.5	38.3	31.5	28.5	26.5	
Norway	32.7	25.8	28.3	22.8	32.7	25.8	28.3	22.8	
Poland	44.0	37.8	25.6	22.0	44.0	37.8	25.6	22.0	
Portugal	38.5	35.0	24.3	20.4	38.5	35.0	24.3	20.4	
Republic of Moldova	45.9	39.3	5.3	3.3	45.9	39.3	5.3	3.3	
Romania	45.2	38.7	23.6	19.4	45.2	38.7	23.6	19.4	
Russian Federation	70.2	65.0	23.2	18.9	70.2	65.0	23.2	18.9	
San Marino	...	...	...	...	...	...	...	...	
Serbia	41.4	37.8	40.4	37.7	41.4	37.8	40.4	37.7	
Slovakia	41.4	34.7	18.5	14.3	41.4	34.7	18.5	14.3	

Slovenia	29.6	26.2	19.9	17.2	29.6	26.2	19.9	17.2
Spain	36.0	32.4	27.7	24.3	36.0	32.4	27.7	24.3
Sweden	19.8	14.9	22.7	17.6	19.8	14.9	22.7	17.6
Switzerland	29.4	22.3	20.3	16.8	29.4	22.3	20.3	16.8
Tajikistan	...	...	...	...	...	...	...	...
The former Yugoslav Republic of Macedonia	...	...	...	...	...	...	...	...
Turkey	53.3	46.4	20.5	15.7	53.3	46.4	20.5	15.7
Turkmenistan	...	...	...	...	...	...	...	...
Ukraine	63.3	57.4	19.3	15.5	63.3	57.4	19.3	15.5
United Kingdom	34.7	27.6	31.1	25.6	34.7	27.6	31.1	25.6
Uzbekistan	24.2	18.9	1.3	0.6	24.2	18.9	1.3	0.6
South-East Asia								
Bangladesh	44.5	39.2	2.9	2.0	41.0	35.5	0.7	0.4
Bhutan	...	...	...	...	...	...	...	...
Democratic People's Republic of Korea	59.5	57.4	...	...	59.5	57.4	...	...
India	30.8	24.9	2.8	1.8	25.8	20.0	0.6	0.3
Indonesia	65.3	57.4	4.2	3.0	61.8	53.0	3.7	2.6
Maldives	44.4	38.0	9.2	7.5	40.6	33.9	7.1	5.6

(continued)

Table 1 (continued)

Country	Smoking any tobacco product [%][a]				Smoking cigarettes [%][b]			
	Males		Females		Males		Females	
	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]	Current[c]	Daily[d]
Myanmar	45.0	34.6	11.7	9.4	42.5	31.8	10.1	7.9
Nepal	29.9	24.0	22.6	21.4	25.2	19.4	22.4	21.2
Sri Lanka	29.9	23.6	2.5	1.5	24.4	18.2	0.4	0.2
Thailand	39.9	29.6	3.4	2.4	37.3	26.9	3.0	2.1
Timor-Leste	…	…	…	…	…	…	…	…
Western Pacific								
! Australia	26.5	21.1	20.3	14.9	26.5	21.1	20.3	14.9
Brunei Darussalam	…	…	…	…	…	…	…	…
Cambodia	31.7	22.0	12.4	9.7	30.3	20.5	10.8	8.4
China	60.8	58.5	4.2	3.8	60.8	58.5	4.2	3.8
Cook Islands	36.5	31.5	20.8	14.2	36.5	31.5	20.8	14.2
Fiji	24.7	19.6	5.1	2.5	24.7	19.6	5.1	2.5
Japan	46.0	42.6	13.7	10.9	46.0	42.6	13.7	10.9
Kiribati	…	…	…	…	…	…	…	…
Lao People's Democratic Republic	62.5	54.2	14.5	11.4	59.2	50.2	12.8	9.9
Malaysia	55.5	44.9	2.5	1.7	52.4	41.4	2.3	1.5
Marshall Islands	…	…	…	…	…	…	…	…
Micronesia (Federated States of)	…	…	…	…	…	…	…	…
Mongolia	44.6	41.6	5.3	4.5	44.6	41.6	5.3	4.5
Nauru	47.2	44.3	53.3	50.5	47.2	44.3	53.3	50.5
New Zealand	25.8	20.7	24.3	19.3	25.8	20.7	24.3	19.3

	Niue	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
	Palau	38.8	34.2	10.1	7.5	38.8	34.2	10.1	7.5
	Papua New Guinea	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
	Philippines	40.7	31.2	9.1	6.8	38.1	28.4	8.0	5.9
	Republic of Korea	53.8	50.7	5.6	4.8	53.8	50.7	5.6	4.8
	Samoa	57.7	55.6	23.8	17.3	57.7	55.6	23.8	17.3
*	Singapore	. . .	23.1	. . .	3.8	. . .	21.1	. . .	3.4
	Solomon Islands	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
	Tonga	61.1	59.3	15.7	10.4	61.1	59.3	15.7	10.4
	Tuvalu	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
	Vanuatu	51.9	49.6	8.0	3.9	51.9	49.6	8.0	3.9
!	Vietnam	44.4	33.9	2.1	1.5	42.0	31.2	1.9	1.3

From MPOWER (World Health Organization 2008), Table 1a

! Data was not validated by country focal point in time for publication of this report

* Current smoking prevalence not validated

. . . Data not available/not reported

[a] Smoking any form of tobacco, including cigarettes, cigars, pipes, bidis, etc.

[b] Smoking manufactured cigarettes

[c] Smoking at the time of the survey, including daily and nondaily smoking

[d] Smoking every day at the time of the survey

24% women), while women smoke much less than men in developing countries (48% men, 7% women) (Group GYTSC 2003). For example, smoking rates among men in China are over 20 times that of women (57 and 2.6%, respectively) (Yang 2008). In the Africa, Eastern Mediterranean, South East Asia, and Western Pacific Regions the ratio of male to female smokers (15 years old or older) is greater than seven, while in the Americas and the Europe Region the ratio is slightly less than two (Group GYTSC 2003). However, a different picture emerges when other tobacco use is considered: the incredible diversity of India, a country with 29 languages each spoken by more than a million native speakers, is reflected in the multiplicity of tobacco products as well (see below).

2.2 Tobacco Use in Youth

The gender gap in smoking is narrowing among adolescents. While among adults in South Africa four times as many men as women smoke (42% vs. 11%), among adolescents aged 11–17 there is only a small difference (39% among girls compared with 55% among boys) (Christofides 2003). Similarly, in China smoking among teenage girls is 7.6% in rural areas and 15.6% in urban areas, contrasted to 2.6% among adult women (Yang 2008); furthermore, the age for smoking initiation in China has dropped from 23 in 1984 to 20 in 1996, and 17 in 2002. The Global Youth Tobacco Survey (GYTS) assessed the use of tobacco products in approximately 750,000 13–15-year-olds from over 140 countries around the world and found that at 70% of sites (82 of 117) there was no statistical difference in tobacco use between girls and boys aged 13–15, and no difference in cigarette smoking in half the sites (Figs. 3–5) (Group GYTSC 2003). This lack of gender difference in use of other tobacco products by youth was seen in more than half the sites in each of the WHO regions except the Eastern Mediterranean. No gender difference was seen for cigarette smoking in at least half the sites in all but two regions, South-East Asia (one third of the sites had no gender difference) and Eastern Mediterranean (all but one of 13 sites showed a gender difference). Globally, boys were twice as likely to smoke as girls, in contrast to men being four times more likely to smoke than women (Warren et al. 2006). Approximately 10% of these young students smoked cigarettes, with the highest rates in Europe (19%) and the lowest in the Eastern Mediterranean (4.9%), which, conversely, had the highest rate (12%) of use of other tobacco products (bidis, kreteks, smokeless tobacco, waterpipes, etc.), which were used by about 10% of these students globally (Warren 2008).

The lowest rate of smoking among these 13–15-year-olds was less than 1% in Goa, while the highest rate, 40%, was found in Coquimbo, Chile. Worldwide, one in five 13–15-year-olds in the GYTS currently used a tobacco product: 14% smoked cigarettes, and 9% used other tobacco products. The lowest rate of use of any tobacco product was also Goa (3.3%) while the highest rate was in India, in Nagaland (63%). Little difference in current smoking between boys and girls was observed in the southern and western regions of India, but significantly more boys smoked than girls in the north, east, central, and northeastern regions (Sinha et al. 2005).

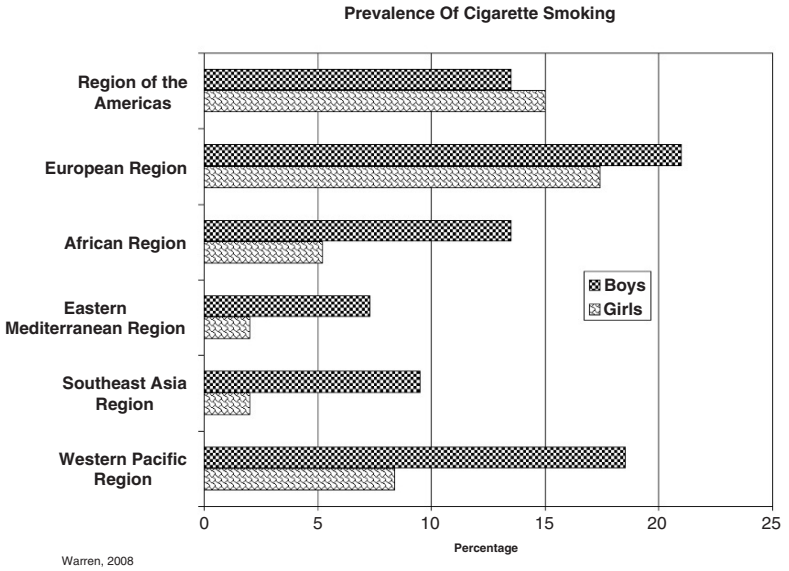

Fig. 3 Prevalence of cigarette smoking among youth (figure from Warren 2008)

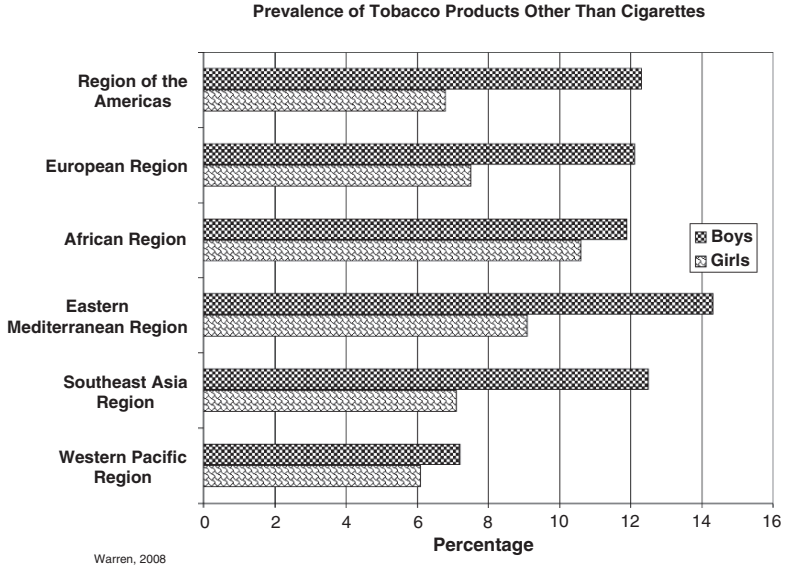

Fig. 4 Prevalence of tobacco products use other than cigarettes among youth (figure from Warren 2008)

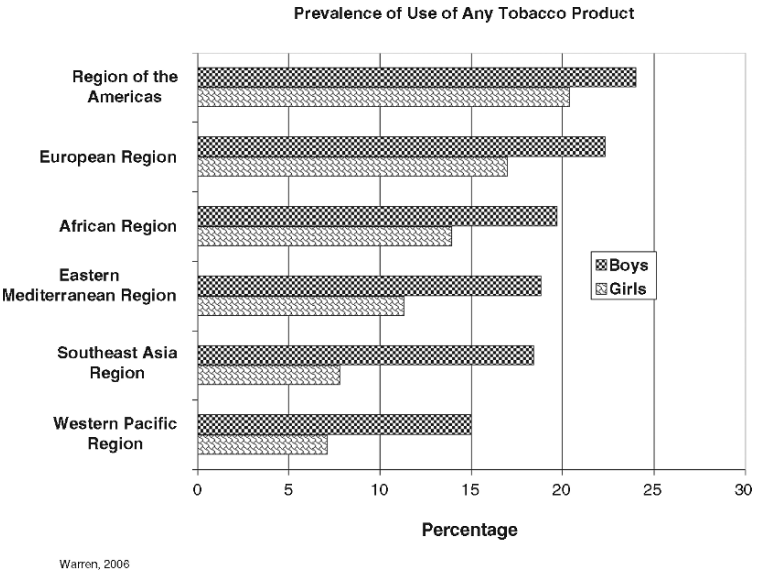

Fig. 5 Prevalence of any tobacco product use among youth (figure from Warren 2008)

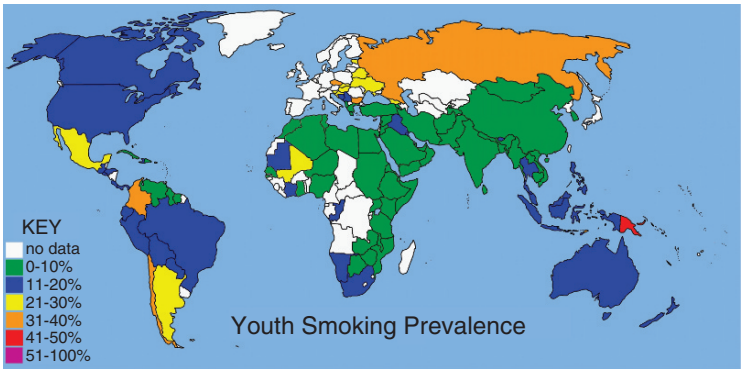

Fig. 6 Youth (13–15-years of age) smoking prevalence (constructed from data in global youth tobacco survey 2003; Group GYTSC 2003)

The smoking rates among young males in sub-Saharan Africa range from 20 to 60% (Townsend et al. 2006a). Among these youth, rates of smoking range from 1.4% in Zimbabwe and 1.5% in Nigeria to 34.4% in Cape Town, South Africa (Townsend et al. 2006b). In Kenya, 7.2% of school-going adolescents smoke cigarettes while 8.5% use other forms of tobacco products (Global Youth Tobacco Survey (GYTS) 2001). The prevalence of smoking among young Ethiopians (15–25 years of age) living in Addis-Ababa was 11.8% for males and 1.1% for females in 1995 (Betre et al. 1997; Rudatsikira et al. 2007)

Figure 6 presents the smoking rates among girls and boys. Cigarette smoking was not as dominant a form of tobacco use as might have been expected among youth

from 120 sites in 76 countries plus the Gaza Strip/West Bank (Group GYTSC 2003). Other tobacco products were used at equal or greater rates as cigarettes in most sites in five of the six WHO regions: Africa, the Americas, Eastern Mediterranean, South-East Asia, and Western Pacific; only in Europe (mostly eastern European sites) were the cigarette smoking rates significantly greater than the rates of usage of other tobacco products.

3 Types of Tobacco Products

Very broadly, tobacco products can be divided into three classes:

1. Those in which tobacco is rolled, combusted and smoked (e.g., cigarettes, bidis, kreteks)
2. Those in which tobacco is heated but not combusted (e.g., water pipes, hookah, nargile)
3. Those in which tobacco is not heated or combusted, i.e., "smokeless tobacco," e.g., snuff, snus, betel quid; these are used orally predominantly, but some are used nasally.

3.1 Combusted Tobacco Products

"Cigarettes are among the most deadly and addictive products ever produced by mankind. When used as intended by their manufacturers, they kill approximately one half of their users"(World Health Organization 2006). Although there are "organic," "natural," and "additive-free" cigarettes, designed to appeal to the health conscious, there is no evidence that these are safer than regular cigarettes. High taxes on manufactured cigarettes have contributed to the increased the popularity of "roll your own" (ryo) cigarettes in the UK and Australia. Worldwide, cigarettes are the most common use of tobacco.

Bidis are small, hand rolled tobacco products wrapped in a tendu or temburni leaf from India and other South East Asian countries, where they are often more popular than manufactured cigarettes. In India, 34% of the tobacco is used in bidis. Variable spices and flavorings are used to reflect regional tastes. Although they contain less tobacco than factory cigarettes, they are inhaled more intensely to maintain the ignition, and so the delivered dose can be higher than that from a cigarette. Despite this, they are increasingly popular in Western countries. For example, 40% of Massachusetts's youth reported smoking bidis at least once.

Cheroots are rolls made from tobacco leaves, while chuttas are a type of cheroot made at home or in cottage industry; 9% of the tobacco produced in India is used to make chuttas. About three billion chutta sticks are made annually in India. Chutta smoking is popular in Andhra Pradesh, Tamil Nadu, and Orissa.

Dhumti is made by rolling a tobacco leaf in the leaf of another plant, forming a conical, cigar-like stick. These are more popular than factory cigarettes in Goa.

Various pipes are used to smoke tobacco, including hooklis (clay pipes, western India), and chillum (a straight, conical pipe held vertically, used in northern India), which may be shared.

Kreteks, or clove cigarettes, typically contain 40% cloves and 60% tobacco, although the ratio varies. These are the most popular form of combusted tobacco use in Indonesia (c. 90%); where over 100 million sticks are manufactured daily. However, they are now being sold globally, especially through the Internet, and the USCDC estimates that as many as 10% of young teenage smokers in the USA may be smoking kreteks.

3.2 Waterpipes

The illusion that the water through which smoke passes cleanses the smoke of toxic chemicals is about 500-years-old. The great physician Abul Fath suggested that smoke "should be passed through a small receptacle of water so that it would be rendered harmless." Tobacco is heated in the "head" of a waterpipe, often using coals or charcoal to heat the tobacco; the smoke passes through water and is inhaled through a tube; the waterpipe may be shared with others, and the act of smoking waterpipes is mostly a social one, either in cafes or with friends or family. However, high levels of toxic chemicals can be inhaled from waterpipe smoking. A recent study of US college students found higher levels of exhaled carbon monoxide after an hour-long waterpipe smoking session than is typical for smokers of two cigarette packs a day (El-Nachef and Hammond 2008).

In some societies the waterpipe is a more acceptable use of tobacco for women than cigarettes. For instance, 2% of the men but 28% of the women from the Darbhanga district of Bihar smoked waterpipes. Waterpipe smoking in India is reportedly declining (Reddy and Gupta 2004).

Over 90% of students at Aga Khan University had smoked waterpipes, but most did not realize that the smoke contained tobacco (Anjum et al. 2008).

A recent study of 646 14–19-year-olds in Pakistan reported that 27% had tried waterpipes, and 17% were current users of waterpipes. Waterpipe smoking was seen by 58% as more socially acceptable than cigarette smoking, and two thirds thought that girls were more comfortable smoking water pipes compared to cigarettes. Waterpipe smoking has become increasingly popular among young adults worldwide, although data are scarce (Maziak et al. 2004); over a quarter of freshman at one US University reported using waterpipes (Smith et al. 2007).

3.3 Noncombusted, or Smokeless Tobacco Products

Smokeless tobacco products are a major form of tobacco addiction in several countries, notably India and South Africa. The tobacco may be chewed, sucked, or applied to the teeth or gums. The products may be manufactured commercially or at home.

Paan (betel quid) with tobacco is commonly used. There are four main ingredients in paan: betel leaf, areca nut, slaked lime, and catechu; tobacco is usually also a component, especially for regular paan users. Sweeteners and flavorings can be added and are often regional. Paan masala is a commercial preparation that is dehydrated and so nonperishable.

In north India there are various regional chewing products that contain tobacco, areca nut and slaked lime. Some of these include mainpuri tobacco and mawa. Khaini is a mixture of dried tobacco and slaked lime that is held in the mouth and used in northern India. Chewing tobacco alone is not common in India.

In India tobacco is used in dental products, despite the fact this is illegal. These products are regional, and used more by women than by men. Some examples include mishri, a blackened roasted tobacco product used to clean teeth; gul, a pyrolysed tobacco product used in eastern India as a dentifrice; baijar, a dry snuff; lal dantmanjan, a red colored dentifrice; and gudhaku, a paste of tobacco and molasses.

The predominant form of smokeless tobacco in Uzbekistan is nasway, which is a mixture of dried tobacco leaves, slaked lime, ash from tree bark, and flavoring and coloring agents; water is added and the mixture is rolled into balls. In 2002, 41% of Uzbek men said they used cigarettes and 38% said they had used nasway; less than 1% of the women used nasway.

In South Africa traditional or home-made products are more commonly used in rural areas while products manufactured by small cottage industries are dominant in urban areas. One of the small smokeless industries was bought by Swedish Match in 1999 and they've continued to manufacture the same products used for both oral and nasal application. Unlike many other countries, nasal use predominates among the 13.2% of black women in South Africa who use smokeless tobacco, 80% nasally and 20% orally. Overall usage is approximately 10%, but reaches 18.6% among black children (Ayo-Yusuf et al. 2004). Only about 1% of South African men use snuff (Ayo-Yusuf et al. 2008).

4 Tobacco Use by Gender and Age

While gender roles and norms in some parts of the world have discouraged women from smoking, smokeless tobacco is more acceptable in some regions (e.g., Africa, India), and waterpipes in others (Middle East). Smokeless tobacco is responsible for four million deaths per year worldwide; half of these are among women; this is predicted to increase to 10 million deaths per year by 2030 (Christofides 2003). In contrast to India, women in the United States are much more likely to smoke cigarettes than to use smokeless tobacco.

For all regions of India, 2.4% of women smoke and 12% chew tobacco. In Goa, 19% of women smoke, mostly bidis (4–13% in various districts); cigarette smoking was negligible. In many areas smokeless tobacco use was more common for women (27% in Goa, 35% in Kerala; virtually no women smoked in Pune district; in Mahrashtra, half of the women used smokeless tobacco and 39% used mishri.

Similarly, in Mumbai only 0.4% of women smoked, but 57% of women 35 and older used smokeless tobacco). The use of chewing tobacco by women varied greatly by region, with less than 1% in several northern states, 5–10% in Andhra Pradesh and Goa, 20–30% Meghalay and Assam, 30–40% in Orissa and Arunachal Pradesh, and 61% in Mizoram.

A similar diversity of tobacco usage was found in the Global Youth Tobacco Survey (GYTS), which was conducted in 26 major Indian states, with 94% of the population of India. The overall prevalence of current use of tobacco products was 17.5% (22% of boys and 10.3% of girls), but the state estimates ranged from 2.3% to 63%. Youth were more likely to use smokeless tobacco (14.6%) than combusted products (8.3%; prevalence of cigarette smoking was 4.2% while other combusted products, e.g., bidis, was 13.6%). Furthermore, the use of tobacco is increasing faster among younger Indian children. Sixth grade boys and girls use tobacco at higher rates than eighth graders (World Health Organization 2006).

For those 10 years or older, 43% rural and 28% urban males are regular tobacco users (10.9 and 4.7% for females). Half to two thirds of males are smokers; 15–20% of female tobacco users were smokers. Generally, rural areas in India have a 50% higher prevalence of tobacco use compared to urban areas.

Although across India only 11% of women use any tobacco product and 1.4% smoke bidis (contrasted to 57% of men using tobacco products and 33% smoking bidis), the rates vary greatly across this vast, diverse country. Although some areas, e.g., Goa, have virtually no bidi smokers among women, and 14% among men, 16% of women in Mizoram smoke bidis, as do 74% of the men there. Women are much more likely to use oral tobacco products; thus in Mizoram 61% of women, and 83% of men, use some tobacco product, contrasted to 4.4% of women in Goa and 28% of men there (IIPS 2007).

5 Tobacco Use in the USA

Tobacco use in the United States has changed dramatically over the past century (Fig. 7). In the early part of the twentieth century two factors contributed to a steep rise in the use of cigarettes: the invention of the automatic cigarette rolling machine, and the provision of free cigarettes to soldiers serving in the military, especially during both World Wars. Whereas in 1900, half the tobacco was consumed as chewing tobacco, and less than 2% as cigarettes, 50 years later per capita consumption of tobacco had doubled and cigarettes were 80% of that. By 1991 cigarettes were 86% of the per capita tobacco consumption, which had fallen to 40% of the 1951 levels and two-thirds of the 1900 levels (Fig. 7). Meanwhile, smokeless tobacco use in the USA increased shortly after the 1964 US Surgeon General's report on the health effects of smoking, until the 1986 Surgeon General's report on the health consequences of using smokeless tobacco. Between 1992 and 2002 smokeless tobacco prevalence declined significantly, from 2.3 to 1.5% (among females the decrease was from 0.43 to 0.16%, while among males the decrease was from 4.8 to 2.9%).

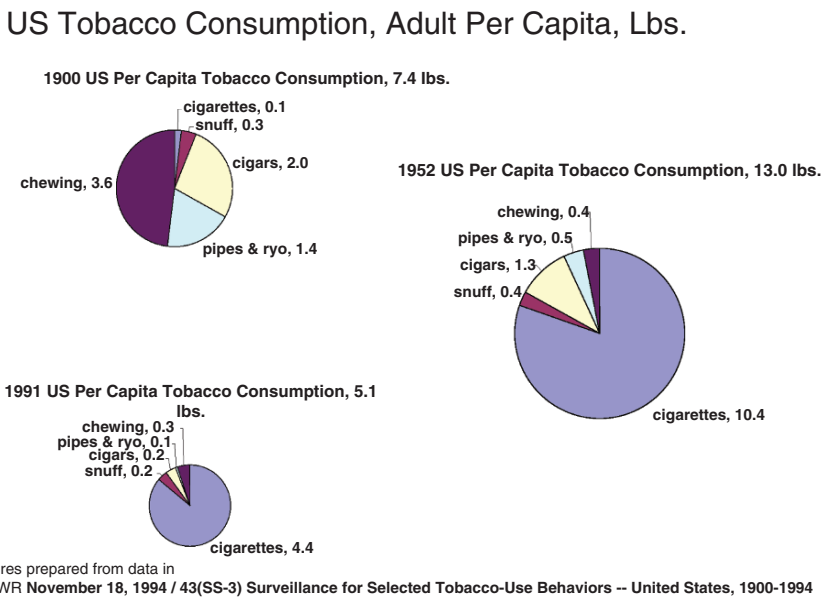

Fig. 7 Changes in US tobacco consumption, 1900–1991 (constructed from data in MMWR 1994; Centers for Disease Control and Prevention 1994)

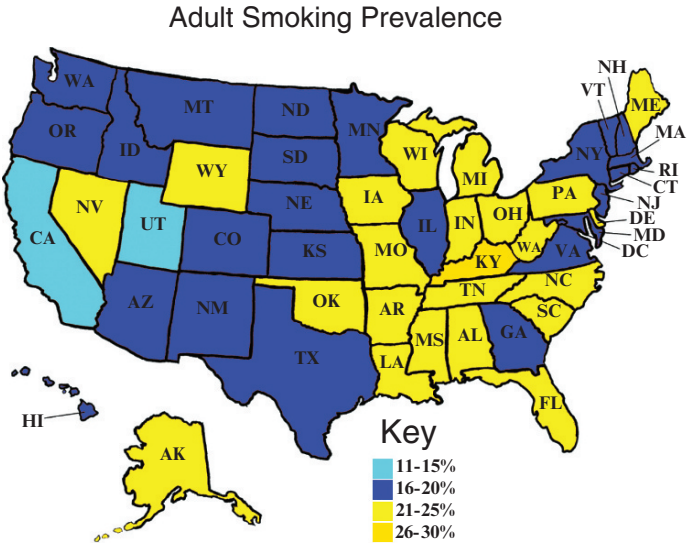

Fig. 8 Adult smoking prevalence in the United States (constructed from data in MMWR 2007; Rock et al. 2007)

The map of smoking prevalence by state (Fig. 8) reveals the range of smoking across the USA; rates are lowest in California, which has had the longest and most active tobacco control program in the country, and Utah, with its large Mormon population.

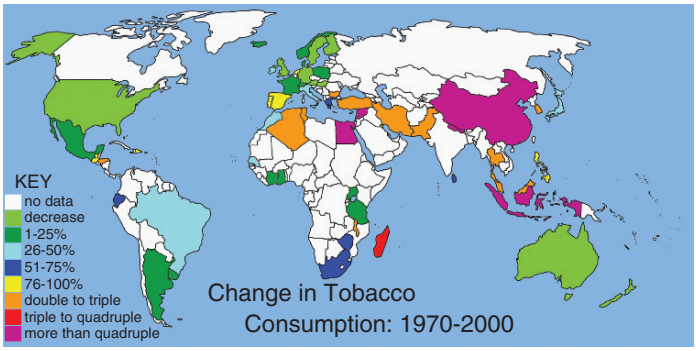

Fig. 9 Change in tobacco consumption, 1970–2000 (constructed from data in MPOWER; World Health Organization 2008)

6 Conclusion

Tobacco kills a third to a half of the people who use it. In India, about a quarter of the deaths among middle aged men are caused by smoking. While many countries in the developed world (notably the USA, Canada, Australia, and the UK) have made significant progress in reducing smoking, tobacco use is increasing rapidly in the developing world, seen as an open market by tobacco companies. (Fig. 9) Women and young children are particular targets, and the success of the marketing strategies is evident in the increasing rates of smoking and other tobacco use in these populations. Without serious attention to this public health threat the current tobacco pandemic will intensify in the next decades.

Acknowledgments My deepest appreciation to Monika Arora and Dr OA Ayo-Yusuf for providing valuable information, to Sylvia Sanchez and Meagan Loftin for manuscript preparation and to Ryan Loomba for the map graphics.

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Nicotine Chemistry, Metabolism, Kinetics and Biomarkers

Neal L. Benowitz, Janne Hukkanen, and Peyton Jacob III

Contents

Abstract Nicotine underlies tobacco addiction, influences tobacco use patterns, and is used as a pharmacological aid to smoking cessation. The absorption, distribution and disposition characteristics of nicotine from tobacco and medicinal products are

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reviewed. Nicotine is metabolized primarily by the liver enzymes CYP2A6, UDP-glucuronosyltransfease (UGT), and flavin-containing monooxygenase (FMO). In addition to genetic factors, nicotine metabolism is influenced by diet and meals, age, sex, use of estrogen-containing hormone preparations, pregnancy and kidney disease, other medications, and smoking itself. Substantial racial/ethnic differences are observed in nicotine metabolism, which are likely influenced by both genetic and environmental factors. The most widely used biomarker of nicotine intake is cotinine, which may be measured in blood, urine, saliva, hair, or nails. The current optimal plasma cotinine cut-point to distinguish smokers from non-smokers in the general US population is 3 ng ml^{-1}. This cut-point is much lower than that established 20 years ago, reflecting less secondhand smoke exposure due to clear air policies and more light or occasional smoking.

1 Introduction

An understanding of the pharmacology of nicotine and how nicotine produces addiction and influences smoking behavior provides a necessary basis for therapeutic advances in smoking cessation interventions. This chapter provides a review of several aspects of the human pharmacology of nicotine. These include the presence and levels of nicotine and related alkaloids in tobacco products, the absorption of nicotine from tobacco products and nicotine medications, the distribution of nicotine in body tissues, the metabolism and renal excretion of nicotine, nicotine and cotinine blood levels during tobacco use or nicotine replacement therapy, and biomarkers of nicotine exposure. For more details and references on the pharmacokinetics and metabolism of nicotine, the reader is referred to Hukkanen et al. (2005c).

2 Nicotine and Related Alkaloids in Tobacco Products

Nicotine (Fig. 1) is a natural ingredient acting as a botanical insecticide in tobacco leaves. It is the principal tobacco alkaloid, occurring to the extent of about 1.5% by weight in commercial cigarette tobacco and comprising about 95% of the total alkaloid content. Oral snuff and pipe tobacco contain concentrations of nicotine similar to cigarette tobacco, whereas cigar and chewing tobacco have only about half the nicotine concentration of cigarette tobacco. An average tobacco rod contains 10–14 mg of nicotine (Kozlowski et al. 1998), and on average about 1–1.5 mg of nicotine is absorbed systemically during smoking (Benowitz and Jacob 1984). Nicotine in tobacco is largely the levorotary (*S*)-isomer; only 0.1–0.6% of total nicotine content is (*R*)-nicotine (Armstrong et al. 1998). Chemical reagents and pharmaceutical formulations of (*S*)-nicotine have a similar content of (*R*)-nicotine (0.1–1.2%) as impurity since plant-derived nicotine is used for their manufacture.

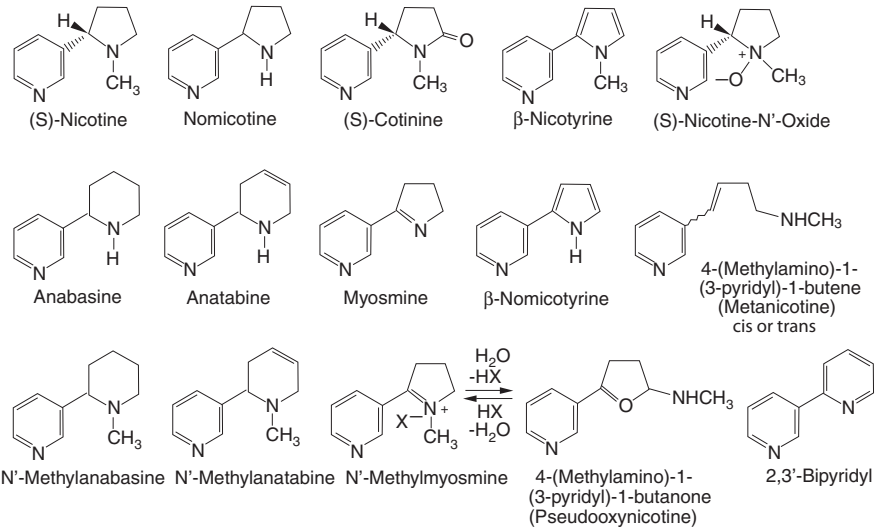

Fig. 1 Structures of tobacco alkaloids. Reprinted from Benowitz and Jacob (1998) with permission of Wiley-Liss, a subsidiary of Wiley

In most tobacco strains, nornicotine and anatabine are the most abundant of minor alkaloids, followed by anabasine (Fig. 1). This order of abundance is the same in cigarette tobacco and oral snuff, chewing, pipe, and cigar tobacco (Jacob et al. 1999). However, nornicotine levels are highest in cigar tobacco, anatabine levels are lowest in chewing tobacco and oral snuff, and anabasine levels are lowest in chewing tobacco (Jacob et al. 1999). Small amounts of the N'-methyl derivatives of anabasine and anatabine are found in tobacco and tobacco smoke. Several of the minor alkaloids are thought to arise by bacterial action or oxidation during tobacco processing rather than by biosynthetic processes in the living plant (Leete 1983). These include myosmine, N'-methylmyosmine, cotinine, nicotyrine, nornicotyrine, nicotine N'-oxide, 2, 3′-bipyridyl, and metanicotine (Fig. 1). Myosmine is found not only in tobacco but also in a variety of foods including nuts, cereals, milk, and potatoes (Tyroller et al. 2002). Also, nicotine is found in low levels in vegetables such as potatoes, tomatoes, and eggplants (Siegmund et al. 1999).

3 Absorption of Nicotine

Nicotine is distilled from burning tobacco and carried proximally on tar droplets (also called particulate matter), which are inhaled. Absorption of nicotine across biological membranes depends on pH. Nicotine is a weak base with a pK_a of 8.0. In its ionized state, such as in acidic environments, nicotine does not rapidly cross membranes. The pH of smoke from flue-cured tobaccos, found in most cigarettes,

is acidic (pH 5.5–6.0). At this pH, nicotine is primarily ionized. As a consequence, there is little buccal absorption of nicotine from flue-cured tobacco smoke, even when it is held in the mouth (Gori et al. 1986). Smoke from air-cured tobaccos, the predominant tobacco used in pipes, cigars, and some European cigarettes, is more alkaline (pH 6.5 or higher) and, considerable nicotine is unionized. Smoke from these products is well absorbed through the mouth (Armitage et al. 1978). It has recently been proposed that the pH of cigarette smoke particulate matter is higher than previously thought, and thus, a larger portion of nicotine would be in the unionized form, facilitating rapid pulmonary absorption (Pankow 2001).

When tobacco smoke reaches the small airways and alveoli of the lung, nicotine is rapidly absorbed. Blood concentrations of nicotine rise quickly during a smoke and peak at the completion of smoking (Fig. 2). The rapid absorption of nicotine from cigarette smoke through the lungs, presumably because of the huge surface area of the alveoli and small airways, and dissolution of nicotine in the fluid of pH 7.4 in the human lung facilitate transfer across membranes. After a puff, high levels of nicotine reach the brain in 10–20 s, faster than with intravenous administration, producing rapid behavioral reinforcement (Benowitz 1990). The rapidity of rise in nicotine levels permits the smoker to titrate the level of nicotine and related effects during smoking, and makes smoking the most reinforcing and dependence-producing form of nicotine administration (Henningfield and Keenan 1993).

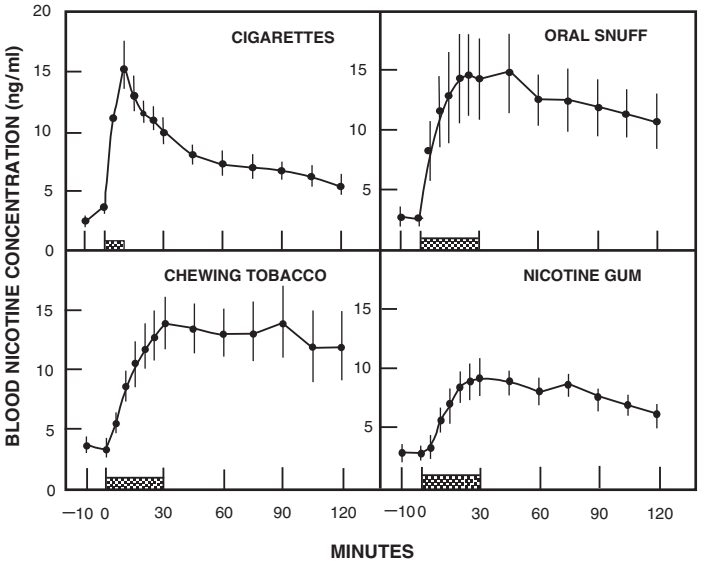

Fig. 2 Blood nicotine concentrations during and after cigarette smoking for 9 min, oral snuff (2.5 g), chewing tobacco (average 7.9 g), and nicotine gum (two 2-mg pieces). Average values for 10 subjects (±SEM). Reprinted from Benowitz et al. (1988) with permission from American Society for Clinical Pharmacology and Therapeutics

The process of cigarette smoking is complex and, as mentioned above, the smoker can manipulate the dose of nicotine and nicotine brain levels on a puff-by-puff basis. Intake of nicotine during smoking depends on puff volume, depth of inhalation, the extent of dilution with room air, and the rate and intensity of puffing (USDHHS 2001). For this reason, machine-determined nicotine yields of cigarettes cannot be used to estimate the dose of nicotine by a smoker (Jarvis et al. 2001). In general, cigarette smokers switching from a higher to a lower-yield cigarette will compensate, i.e., will change their smoking pattern to gain more nicotine (USDHHS 2001).

Chewing tobacco and snuff are buffered to alkaline pH to facilitate absorption of nicotine through oral mucosa. Although absorption through cell membranes is rapid for these more alkaline tobacco products, the rise in the brain nicotine level is slower than with smoking (Fig. 2). Concentrations of nicotine in the blood rise gradually with the use of smokeless tobacco and plateau at about 30 min, with levels persisting and declining only slowly over 2 h or more (Benowitz et al. 1988).

Various formulations of nicotine replacement therapy (NRT), such as nicotine gum, transdermal patch, nasal spray, inhaler, sublingual tablets, and lozenges, are buffered to alkaline pH to facilitate absorption of nicotine through cell membranes. Absorption of nicotine from all NRTs is slower and the increase in nicotine blood levels is more gradual than from smoking. This slow increase in blood and especially in brain levels results in low abuse liability of NRTs (West et al. 2000). Only nasal spray provides a rapid delivery of nicotine that is closer to the rate of nicotine delivery achieved with smoking (Gourlay and Benowitz 1997; Guthrie et al. 1999). The absolute dose of nicotine absorbed systemically from nicotine gum is much less than the nicotine content of the gum, in part, because considerable nicotine is swallowed with subsequent first-pass metabolism (Benowitz et al. 1987). Some nicotine is also retained in chewed gum. A portion of the nicotine dose is swallowed and subjected to first-pass metabolism when using other NRTs, inhaler, sublingual tablets, nasal spray, and lozenges. Bioavailability for these products with absorption mainly through the mucosa of the oral cavity and a considerable swallowed portion is about 50–80%.

Nicotine base is well absorbed through skin. That is the reason for the occupational risk of nicotine poisoning (green tobacco sickness) in tobacco harvesters who are exposed to wet tobacco leaves (McBride et al. 1998). That is also the basis for transdermal delivery technology. Currently in the United States several different nicotine transdermal systems are marketed. All are multilayer patches. The rate of release of nicotine into the skin is controlled by the permeability of the skin, rate of diffusion through a polymer matrix, and/or rate of passage through a membrane in the various patches. Rates of nicotine delivery and plasma nicotine concentrations vary among different transdermal systems (Fant et al. 2000). In all cases, there is an initial lag time of about 1 h before nicotine appears in the bloodstream, and there is continued systemic absorption (about 10% of the total dose) after the patch is removed, the latter due to residual nicotine in the skin.

4 Distribution of Nicotine in Body Tissues

After absorption, nicotine enters the bloodstream where, at pH 7.4, it is about 69% ionized and 31% unionized. Binding to plasma proteins is less than 5% (Benowitz et al. 1982a). The drug is distributed extensively to body tissues with a steady-state volume of distribution averaging 2.6 L/Kg. Based on human autopsy samples from smokers, the highest affinity for nicotine is in the liver, kidney, spleen, and lung and lowest in adipose tissue. In skeletal muscle, concentrations of nicotine and cotinine are close to that of whole blood. Nicotine binds to brain tissues with high affinity, and the receptor binding capacity is increased in smokers compared with nonsmokers (Breese et al. 1997; Perry et al. 1999). Increase in the binding is caused by a higher number of nicotinic cholinergic receptors in the brain of the smokers. Nicotine accumulates markedly in gastric juice and saliva (Lindell et al. 1996). Gastric juice/plasma and saliva/plasma concentration ratios are 61 and 11 with transdermal nicotine administration, and 53 and 87 with smoking, respectively (Lindell et al. 1996). Accumulation is caused by ion-trapping of nicotine in gastric juice and saliva. Nicotine also accumulates in breast milk (milk/plasma ratio 2.9) (Dahlstrom et al. 1990). Nicotine crosses the placental barrier easily, and there is evidence for accumulation of nicotine in fetal serum and amnionic fluid in slightly higher concentrations than in maternal serum (Dempsey and Benowitz 2001).

The time course of nicotine accumulation in the brain and in other body organs and the resultant pharmacologic effects are highly dependent on the route and rate of dosing. Smoking a cigarette delivers nicotine rapidly to the pulmonary venous circulation, from which it moves quickly to the left ventricle of the heart and to the systemic arterial circulation and brain. The lag time between a puff of a cigarette and nicotine reaching the brain is 10–20 s. Although delivery of nicotine to the brain is rapid, there is nevertheless significant pulmonary uptake and some delayed release of nicotine as evidenced by pulmonary positron emission tomography data and the slow decrease in arterial concentrations of nicotine between puffs. (Rose et al. 1999) Nicotine concentrations in arterial blood after smoking a cigarette can be quite high, reaching up to 100 ng ml^{-1}, but usually ranging between 20 and 60 ng ml^{-1} (Gourlay and Benowitz 1997; Henningfield and Keenan 1993; Lunell et al. 2000; Rose et al. 1999). The usual peak arterial nicotine concentration after the first puff is lower, averaging 7 ng ml^{-1}. As high as tenfold arterial/venous nicotine concentration ratios have been measured (Henningfield et al. 1993), but the mean ratio is typically around 2.3–2.8 (Gourlay and Benowitz 1997; Rose et al. 1999). The rapid rate of delivery of nicotine by smoking (or intravenous injection, which presents similar distribution kinetics) results in high levels of nicotine in the central nervous system with little time for development of tolerance. The result is a more intense pharmacologic action. The short time interval between puffing and nicotine entering the brain also allows the smoker to titrate the dose of nicotine to a desired pharmacologic effect, further reinforcing drug self-administration and facilitating the development of addiction.

5 Metabolism of Nicotine

5.1 Pathways of Nicotine and Cotinine Metabolism

Nicotine is extensively metabolized to a number of metabolites (Fig. 3) by the liver. Six primary metabolites of nicotine have been identified. Quantitatively, the most important metabolite of nicotine in most mammalian species is the lactam derivative, cotinine. In humans, about 70–80% of nicotine is converted to cotinine. This transformation involves two steps. The first is mediated primarily by CYP2A6 to produce nicotine-$\Delta^{1'(5')}$-iminium ion, which is in equilibrium with 5′-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase. Nicotine iminium ion has received considerable interest since it is an alkylating agent and, as such, could play a role in the pharmacology of nicotine (Shigenaga et al. 1988).

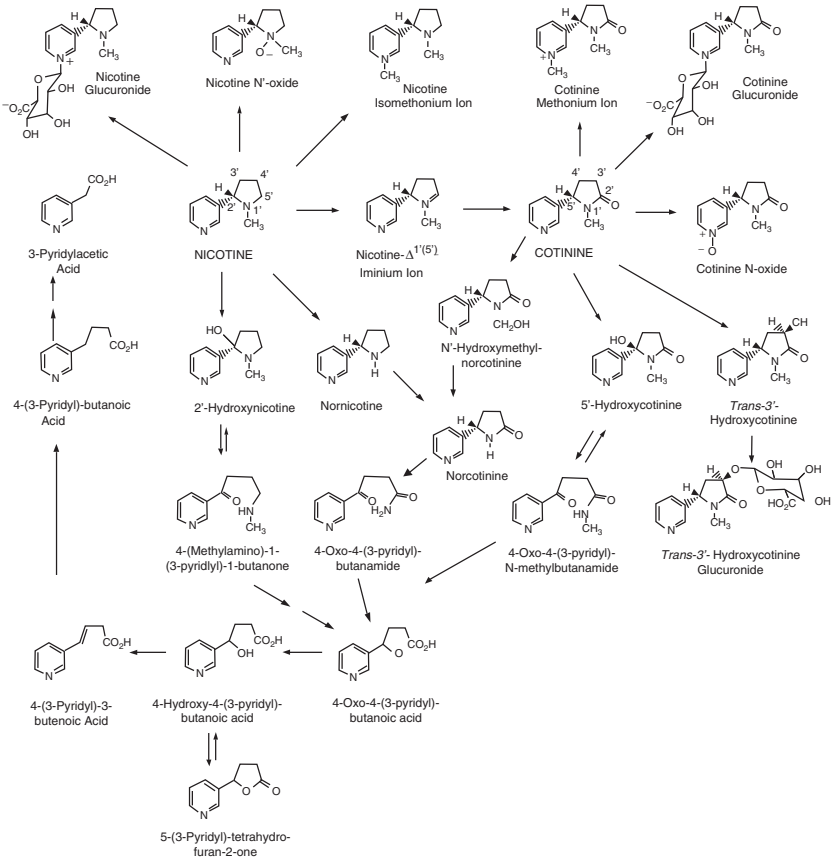

Fig. 3 Pathways of nicotine metabolism. Reprinted with permission from Hukkanen et al. 2005c

Nicotine N'-oxide is another primary metabolite of nicotine, although only about 4–7% of nicotine absorbed by smokers is metabolized via this route (Benowitz et al. 1994). The conversion of nicotine to nicotine N'-oxide involves a flavin-containing monooxygenase 3 (FMO3), which results in formation of both possible diastereomers, the 1′-(*R*)-2′-(*S*)-*cis* and 1′-(*S*)-2′-(*S*)-*trans*-isomers in animals (Cashman et al. 1992; Park et al. 1993). In humans, this pathway is highly selective for the *trans*-isomer (Cashman et al. 1992). Only the *trans*-isomer of nicotine N'-oxide was detected in urine after administration of nicotine by intravenous infusion, transdermal patch or smoking (Park et al. 1993). It appears that nicotine N'-oxide is not further metabolized to any significant extent, except by reduction back to nicotine in the intestines, which may lead to recycling nicotine in the body.

In addition to oxidation of the pyrrolidine ring, nicotine is metabolized by two nonoxidative pathways, methylation of the pyridine nitrogen giving nicotine isomethonium ion (also called *N*-methylnicotinium ion) and glucuronidation.

Nicotine glucuronidation results in an *N*-quaternary glucuronide in humans (Benowitz et al. 1994). This reaction is catalyzed by uridine diphosphate-glucuronosyltransferase (UGT) enzyme(s) producing (*S*)-nicotine-*N*-β-glucuronide. About 3–5% of nicotine is converted to nicotine glucuronide and excreted in urine in humans.

Oxidative *N*-demethylation is frequently an important pathway in the metabolism of xenobiotics, but this route is, in most species, a minor pathway in the metabolism of nicotine. Conversion of nicotine to nornicotine in humans has been demonstrated. We found that small amounts of deuterium-labeled nornicotine are excreted in the urine of smokers administered deuterium-labeled nicotine (Jacob and Benowitz 1991). Metabolic formation of nornicotine from nicotine has also been reported (Neurath et al. 1991). Nornicotine is a constituent of tobacco leaves. However, most urine nornicotine is derived from metabolism of nicotine with less than 40% coming directly from tobacco, as estimated from the difference in nornicotine excretion in smokers during smoking and transdermal nicotine treatment (0.65 and 0.41%, respectively) (Benowitz et al. 1994). A new cytochrome P450-mediated metabolic pathway for nicotine metabolism was reported by Hecht et al. (2000). 2′-Hydroxylation of nicotine was shown to produce 4-(methylamino)-1-(3-pyridyl)-1-butanone with 2′-hydroxynicotine as an intermediate. 2′-Hydroxynicotine also yields nicotine-$\Delta^{1'(2')}$-iminium ion. 4-(methylamino)-1-(3-pyridyl)-1-butanone is further metabolized to 4-oxo-4-(3-pyridyl)butanoic acid and 4-hydroxy-4-(3-pyridyl)butanoic acid. The new pathway is potentially significant since 4-(methylamino)-1-(3-pyridyl)-1-butanone can be converted to carcinogenic NNK. However, endogenous production of NNK from nicotine has not been detected in humans or rats (Hecht et al. 1999a).

Although on average about 70–80% of nicotine is metabolized via the cotinine pathway in humans, only 10–15% of nicotine absorbed by smokers appears in the urine as unchanged cotinine (Benowitz et al. 1994). Six primary metabolites of cotinine have been reported in humans: 3′-hydroxycotinine (McKennis et al. 1963; Neurath et al. 1987), 5′-hydroxycotinine (also called allohydroxycotinine) (Neurath 1994), which exists in tautomeric equilibrium with the open chain derivative

4-oxo-4-(3-pyridyl)-*N*-methylbutanamide, cotinine *N*-oxide, cotinine methonium ion, cotinine glucuronide, and norcotinine (also called demethylcotinine).

3′-Hydroxycotinine is the main nicotine metabolite detected in smokers' urine. It is also excreted as a glucuronide conjugate (Benowitz et al. 1994). 3′-Hydroxycotinine and its glucuronide conjugate account for 40–60% of the nicotine dose in urine (Benowitz et al. 1994; Byrd et al. 1992). The conversion of cotinine to 3′-hydroxycotinine in humans is highly stereoselective for the *trans*-isomer, as less than 5% is detected as *cis*-3′-hydroxycotinine in urine (Jacob et al. 1990; Voncken et al. 1990). While nicotine and cotinine conjugates are *N*-glucuronides, the only 3′-hydroxycotinine conjugate detected in urine is *O*-glucuronide (Byrd et al. 1994).

Quantitative aspects of the pattern of nicotine metabolism have been elucidated fairly well in people (Fig. 4). Approximately 90% of a systemic dose of nicotine can be accounted for as nicotine and metabolites in urine (Benowitz et al. 1994). Based on studies with simultaneous infusion of labeled nicotine and cotinine, it has been determined that 70–80% of nicotine is converted to cotinine (Benowitz and Jacob 1994). About 4–7% of nicotine is excreted as nicotine *N*′–oxide and 3–5% as nicotine glucuronide (Benowitz et al. 1994; Byrd et al. 1992). Cotinine is excreted unchanged in urine to a small degree (10–15% of the nicotine and metabolites in urine). The remainder is converted to metabolites, primarily *trans*–3′–hydroxycotinine (33–40%), cotinine glucuronide (12–17%), and *trans*–3′–hydroxycotinine glucuronide (7–9%).

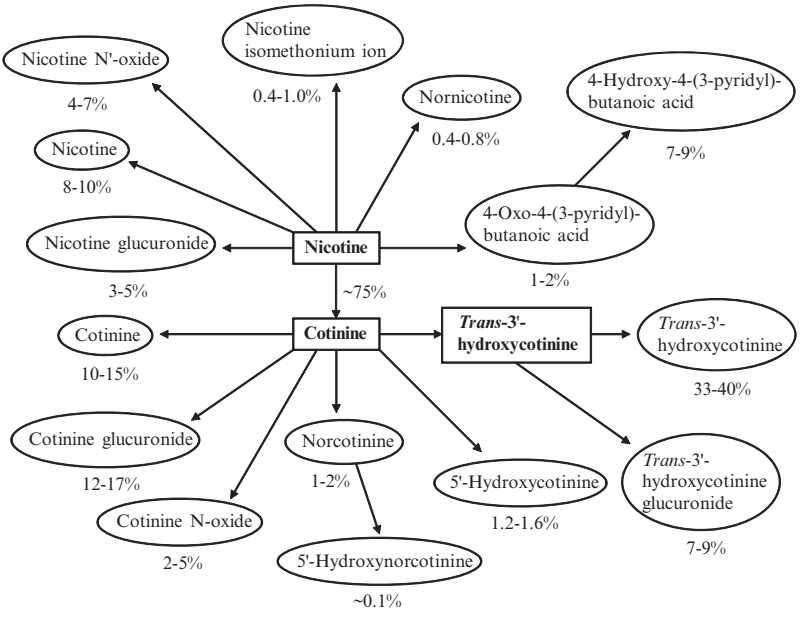

Fig. 4 Quantitative scheme of nicotine metabolism, based on estimates of average excretion of metabolites as percent of total urinary nicotine. Reprinted with permission from Hukkanen et al. 2005c

5.2 Rates of Nicotine and Cotinine Metabolism

The rate of metabolism of nicotine can be determined by measuring blood levels after administration of a known dose of nicotine (Table 1) (Hukkanen et al. 2005c). Total clearance of nicotine averages about 1200 ml min^{-1}. Nonrenal clearance represents about 70% of liver blood flow. Assuming most nicotine is metabolized by the liver, this means that about 70% of the drug is extracted from blood in each pass through the liver.

The metabolism of cotinine is much slower than that of nicotine. Cotinine clearance averages about 45 ml min^{-1}. Clearance of (3′*R*, 5′*S*)-*trans*-3′-hydroxycotinine is also quite slow – about 82 ml min^{-1}.

5.3 Use of the Nicotine Metabolite Ratio

The 3′-hydroxycotinine/cotinine ratio (3HC/cotinine) in plasma and saliva has been evaluated as a non-invasive probe for CYP2A6 activity (Dempsey et al. 2004). The ratio was highly correlated with oral clearance of nicotine and the oral clearance and half-life of cotinine. Correlation coefficients of oral nicotine and cotinine clearances with plasma 3′-hydroxycotinine/cotinine ratios were 0.78 and 0.63, respectively, at 6 h after oral nicotine dosing.

The availability of a phenotypic marker of CYP2A6 activity is important because there is wide variability in nicotine clearance among people with wild-type CYP2A6 genes and only a small proportion of the genetic variability in nicotine clearance can be explained by known CYP2A6 gene variants, at least in whites (Swan et al. 2005). The 3′-hydroxycotinine/cotinine ratio can be used to phenotype nicotine metabolism and CYP2A6 enzyme in smokers while smoking their usual cigarettes (Johnstone et al. 2006; Kandel et al. 2007; Lerman et al. 2006; Patterson et al. 2008). The 3HC/cotinine ratio has been studied as predictor of response to pharmacotherapy.

In one trial, where transdermal nicotine and nicotine nasal spray were compared, the nicotine metabolite ratio (derived from nicotine taken in from tobacco) was shown to be a strong predictor of smoking cessation, both at the end of treatment and in 6 months, in people treated with transdermal nicotine but not nicotine nasal spray (Lerman et al. 2006). In patients treated with transdermal nicotine, slow metabolizers had better cessation response and higher plasma nicotine concentration while using the patch than faster metabolizers, suggesting that higher nicotine levels might be responsible for a better cessation outcome. In contrast, smokers treated with nicotine nasal spray showed no difference in plasma nicotine concentration as a function of the rate of nicotine metabolism, consistent with the idea that nicotine taken in from the spray is titrated by the smoker to the desired effect. However, another recent trial examined the association between the nicotine metabolite ratio and response to bupropion therapy (Patterson et al. 2008). Faster metabolism of nicotine was associated with lower success rate in quitting in a placebo-treated group; but among smokers receiving bupropion, the rate of nicotine metabolism

Table 1 Nicotine absorption pharmacokinetics of different forms of nicotine administration in single doses (modified from Hukkanen et al. 2005c)

Type of nicotine administration[a]	C_{max}[b] ng ml^{-1}	T_{max}[b,c] min	Bioavailability %
Smoking (one cigarette, 5 min)	15–30 (venous)	5–8 (venous)	80–90 (of inhaled
(~2 mg/cigarette[d])	20–60 (arterial)	3–5 (arterial)	nicotine)
Intravenous ~5.1 mg	30 (venous)	30 (venous)	100
(60 µg/kg, 30 min)	50 (arterial)	30 (arterial)	
Nasal spray 1 mg	5–8 (venous)	11–18 (venous)	60–80
	10–15 (arterial)	4–6 (arterial)	
Gum (30 min, total dose in gum)			
2 mg	6–9	30	78
4 mg	10–17	30	55
Inhaler 4 mg released	8.1	30	51–56
(one 10 mg cartridge, 20 min)			
Lozenge (20–30 min)			
2 mg	4.4	60	50
4 mg	10.8	66	79
Sublingual tablet 2 mg (20–30 min)	3.8	~60	65
Tooth patch 2 mg	~3.2	~120	
Transdermal patch (labeled dose)			
15 mg/16 h (Nicotrol)	11–14	6–9 h	75–100
14 mg/24 h (Nicoderm)	11–16	4–7 h	
21 mg/24 h (Nicoderm)	18–23	3–7 h	68
21 mg/24 h (Habitrol)	12–21	9–12 h	82
Subcutaneous injection 2.4 mg	15	25	100
Oral capsule 3–4 mg	6–8	90	44
Oral slow-release capsule	2.2	7.5 h	
(colonic absorption) 6 mg			
Oral solution			
2 mg	4.7	51	
~3.0 mg (45 µg/kg)	2.9	66	20
Enema			
~3.5 mg (45 µg/kg)	2.3–3.1	20–80	15–25
6 mg	6–9	45	

[a]Products in italics are currently marketed in the United States
[b]C_{max} and T_{max} values are for peripheral venous blood unless otherwise indicated
[c]T_{max} values are measured from the start of the administration
[d]Estimated dose of 2 mg of nicotine per cigarette is higher than the usual 1–1.5 mg per cigarette since nicotine absorption from smoking a single cigarette was studied after at least overnight abstinence from smoking in these studies

had no differential effect. Bupropion is not metabolized by CYP2A6. Therefore, the findings of the Patterson study are consistent with the idea that rapid metabolizers of nicotine are generally more dependent and have a harder time quitting than do slow metabolizers. The mechanisms of such a relationship have not been proven, but may include more severe withdrawal symptoms and/or a different type of nicotine reinforcement related to more rapid loss of tolerance in fast metabolizers.

Various enzymes involved in nicotine metabolism and their genetics are described in detail in the chapter by Mwenifumbo and Tyndale in this volume.

6 Factors Influencing Nicotine Metabolism

There is considerable inter individual variability in the rate of elimination of nicotine and cotinine in people (Swan et al. 2005). Besides genetic variations discussed by Mwenifumbo and Tyndale, a number of factors that might explain individual variability have been studied.

6.1 Physiological Influences

6.1.1 Diet and Meals

An implication of the high degree of hepatic extraction is that clearance of nicotine should be dependent on liver blood flow. Thus, physiological events, such as meals, posture, exercise, or drugs perturbing hepatic blood flow, are predicted to affect the rate of nicotine metabolism. Meals consumed during a steady state infusion of nicotine result in a consistent decline in nicotine concentrations, the maximal effect seen 30–60 min after the end of a meal (Gries et al. 1996; Lee et al. 1989). Hepatic blood flow increases about 30% and nicotine clearance increases about 40% after a meal.

Menthol is widely used as a flavorant in foods, mouthwash, toothpaste, and cigarettes. A moderate inhibition of CYP2A6-mediated nicotine metabolism in human liver microsomes by menthol and various related compounds has been reported (MacDougall et al. 2003). This is supported by a crossover study in people, showing that mentholated cigarette smoking significantly inhibits metabolism of nicotine to cotinine and nicotine glucuronidation when compared to smoking nonmentholated cigarettes (Benowitz et al. 2004).

Grapefruit juice inhibits CYP2A6, as evidenced by inhibition of coumarin metabolism in people (Runkel et al. 1997). Grapefruit juice has been shown to inhibit the metabolism of nicotine to cotinine in nonsmokers who were given nicotine orally, with evidence of a greater effect with larger doses of grapefruit juice (Hukkanen et al. 2006). Grapefruit juice also increased renal clearance of nicotine and cotinine by an unknown mechanism. Grapefruit juice had no signficant effect on overall exposure to nicotine (area under the plasma concentration–time curve) because the effects of slowed metabolism were offset by the effects on increased renal clearance. Whether the effects of grapefruit juice on nicotine levels in users of tobacco are significant has not been investigated. Consumption of watercress enhances the formation of nicotine glucuronide, cotinine glucuronide, and 3′-hydroxycotinine glucuronide in smokers (Hecht et al. 1999b). Watercress has no effect on the excretion of nicotine, cotinine, and 3′-hydroxycotinine in smokers. Thus, watercress may induce some UGT enzymes involved in nicotine metabolism, but has no effect on CYP2A6-mediated nicotine metabolism.

6.1.2 Age

Clearance of nicotine is decreased in the elderly (age >65) compared to adults (Molander et al. 2001). Total clearance was lower by 23%, and renal clearance lower by 49% in the elderly compared to young adults. Lower nicotine metabolism in the elderly may be contributed to by reduced liver blood flow, since no decrease in CYP2A6 protein levels or nicotine metabolism in liver microsomes due to age has been detected (Messina et al. 1997). No differences in steady-state nicotine plasma levels or estimated plasma clearance values were detected in three age groups (18–39, 40–59, and 60–69 years) using patches with the same nicotine content (Gourlay and Benowitz 1996). The volume of distribution of nicotine is lower in older subjects due to a decrease in lean body mass (Molander et al. 2001).

Neonates have diminished nicotine metabolism, as demonstrated by a nicotine half-life of three to four times longer in newborns exposed to tobacco smoke than in adults (Dempsey et al. 2000). Cotinine half-life is reported to be similar in neonates, older children, and adults in two studies (Dempsey et al. 2000; Leong et al. 1998). Other studies found that the half-life of urine cotinine was about three times longer in children less than one year old than to the cotinine half-life in adults (Collier et al. 1994). Urine cotinine half-life can be influenced by variations in urine volume and excretion of creatinine. The study by Dempsey et al. was the only one in which the half-life of cotinine was calculated based on both the blood and urine cotinine concentrations (Dempsey et al. 2000). In that study, both the blood and urine half-lives were similar to adult values, supporting the notion that neonates have the same cotinine half-life as older children and adults.

Why nicotine has a much longer half-life in neonates than in adults, whereas the cotinine half-life is essentially the same in newborns and adults, might partially be explained by differing sensitivities of nicotine and cotinine clearances to changes in hepatic blood flow. As a drug with a high extraction ratio, the clearance of nicotine is influenced by changes in hepatic blood flow, whereas clearance of cotinine with low extraction ratio is more dependent on changes in intrinsic clearance, i.e., amount and activity of metabolic enzymes. Studies in newborn animals, mainly sheep, have shown that hepatic blood flow is low immediately after delivery because of the loss of the umbilical venous blood supply and the patency of ductus venosus (Gow et al. 2001). Hepatic blood flow (ml^{-1} min^{-1} mg of liver) rises to adult levels within the first week, due to increased blood flow in the portal vein and gradual closure of ductus venosus, which is complete by the eighteenth day in human neonates. This would mean that nicotine clearance should rise and the nicotine half-life shorten within the first couple of weeks as hepatic blood flow increases. Another explanation could be that nicotine and cotinine are metabolized mainly by enzymes other than CYP2A6 in neonates. However, neonates have only slightly lower amounts of CYP2A6, CYP2D6, and CYP2E1 protein in liver microsomes, whereas the CYP2B6 amount is clearly diminished in neonates compared to adults and older children (Tateishi et al. 1997).

6.1.3 Chronopharmacokinetics of Nicotine

During sleep, hepatic blood flow declines and nicotine clearance falls correspondingly. Blood nicotine levels rise during constant infusion at night. Nicotine clearance varies by approximately 17% (from peak to trough) with a minimum between 6 p.m. and 3 a.m. Thus, the day/night variation and meal effects of nicotine clearance result in circadian variations in plasma concentrations during constant dosing of nicotine (Gries et al. 1996).

6.1.4 Gender Related Differences in Nicotine Metabolism

Differences between Men and Women

A twin study with intravenous infusions of both nicotine and cotinine clearly shows that nicotine and cotinine clearances are higher in women than in men; oral contraceptive use further accelerates nicotine and cotinine clearances in women (Benowitz et al. 2006). Nicotine clearance and cotinine clearance were 13 and 24% higher, respectively, in women not using oral contraceptives than in men. Oral contraceptive use induced increases in nicotine and cotinine clearance by 28 and 30%, respectively, compared to women not using oral contraceptives. The gender difference was also detected in recent studies on smokers, showing that the ratio of 3HC/cotinine in blood or urine is significantly higher in women indicating faster metabolism in women than men (Johnstone et al. 2006; Kandel et al. 2007).

Pregnancy and Menstrual Cycle

Pregnancy has a marked inducing effect in nicotine and especially cotinine clearance. Clearance is increased by 60 and 140% for nicotine and cotinine, respectively, in pregnancy compared to postpartum (Dempsey et al. 2002). Nicotine is a rapidly cleared drug with a high affinity for CYP2A6 and its rate of clearance is primarily controlled by hepatic blood flow, while the rate of cotinine clearance is primarily determined by the activity of metabolizing enzymes in the liver. The finding that in pregnancy cotinine clearance is increased more than nicotine clearance indicates that the increase in clearance is most likely caused by induction of CYP2A6, and not by an increase in hepatic blood flow. A study comparing women during pregnancy and again postpartum, found that the mean salivary cotinine concentration per cigarette was higher when not pregnant (3.5 ng ml^{-1} vs. 9.9 ng ml^{-1}), consistent with higher cotinine clearance during pregnancy (Rebagliato et al. 1998). Pregnant smokers had substantially lower levels of serum nicotine than expected when standardized for their nicotine intake compared to population-based values (Selby et al. 2001). Nicotine and cotinine glucuronidation is induced by pregnancy, while 3′-hydroxycotinine glucuronidation is not (Dempsey et al. 2002). Menstrual

cycle (follicular phase vs. luteal phase) has no effect on nicotine and cotinine pharmacokinetics in healthy nonsmoking women (Hukkanen et al. 2005b). Pregnancy also increases the rate of formation of nicotine N'-oxide, indicating induction of the enzyme, flavin-containing monooxygenase 3 (Hukkanen et al. 2005a).

The above-mentioned results show that gender has substantial effects on nicotine and cotinine metabolism. Higher metabolism of nicotine and cotinine is detected in women than in men, in users of oral contraceptives than in women not using oral contraceptives, and in pregnant women than in the same subjects postpartum. Furthermore, the inducing effect has a dose–response relationship; gender differences are relatively small, oral contraceptive use further induces metabolism in women, and pregnancy shows the most striking induction compared to postpartum. Changes in clearance appear to be related to the amount of sex hormones present; women have higher concentrations of estrogens and progesterone than men do, oral contraceptive users have higher concentrations of these hormones than women not using oral contraceptives, and pregnancy results in the highest concentrations of circulating sex hormones. These results suggest that CYP2A6 activity is induced by sex hormones and there is recent in-vitro evidence for the induction of human CYP2A6 by estrogen acting on the estrogen receptor (Higashi et al. 2007).

Kidney Disease

Kidney failure not only decreases renal clearance of nicotine and cotinine, but also metabolic clearance of nicotine (Molander et al. 2000). Metabolic clearance of nicotine is reduced by 50% in subjects with severe renal impairment compared to healthy subjects. It is speculated that accumulation of uremic toxins may inhibit CYP2A6 activity or downregulate CYP2A6 expression in liver. Hepatic metabolism of several drugs is reduced in kidney failure, mainly via downregulation of CYP enzymes and/or inhibition of transporters (Nolin et al. 2003).

6.2 Medications

6.2.1 Inducers

A few drugs have been shown to induce CYP2A6 in human primary hepatocyte culture. These include prototypical inducers rifampicin, dexamethasone, and phenobarbital, although there is wide interindividual variability in response (Madan et al. 2003; Meunier et al. 2000; Rae et al. 2001). Rifampicin was also shown to inhibit CYP2A6 activity as measured by coumarin 7-hydroxylase (Xia et al. 2002). Thus the presence of rifampin may inhibit while chronic administration of rifampin may induce CYP2A6. That might explain the highly variable effects on CYP2A6 induction seen in studies with rifampicin.

There is evidence for the induction of CYP2A6 in vivo by phenobarbital and other anticonvulsant drugs. Two-day treatment with phenobarbital (100 mg per day p.o.) prior to a liver biopsy resulted in induction of metabolism of nicotine to cotinine in hepatocytes (Kyerematen et al. 1990). Liver microsomes from phenobarbital-treated patients have higher amounts of CYP2A6 protein than microsomes from untreated patients (Cashman et al. 1992). A recent study showed that the antimalarial drug artemisinin significantly altered the pharmacokinetics of both nicotine and coumarin, suggesting induction of CYP2A6. (Asimus et al. 2008).

As mentioned earlier, nicotine and cotinine clearances are higher in women using oral contraceptives than in women not using oral contraceptives (Benowitz et al. 2006). Oral contraceptive use induced nicotine and cotinine clearances by 28 and 30%, respectively. A previous small-scale study with caffeine phenotyping of CYP2A6 activity showed a 22% increase in CYP2A6 activity in oral contraceptive users compared to women not using contraceptives (Krul and Hageman 1998).

6.2.2 Inhibitors

Several compounds are inhibitors of CYP2A6-mediated nicotine metabolism in vitro, including methoxsalen (8-methoxypsoralen), tranylcypromine, tryptamine and coumarin (Le Gal et al. 2003; MacDougall et al. 2003; Nakajima et al. 1996; Zhang et al. 2001). Raloxifene is a potent inhibitor of aldehyde oxidase and it has been shown to inhibit the formation of cotinine from nicotine-$\Delta^{1'(5')}$-iminium ion in human liver cytosol (Obach 2004).

Only methoxsalen (used in the photochemotherapy of psoriasis) and tranylcypromine (a monoamine oxidase inhibitor) have been demonstrated to inhibit nicotine metabolism in people (Sellers et al. 2000, 2003). These compounds are only moderately specific for CYP2A6; methoxsalen is also a potent inhibitor of CYP1A2, and tranylcypromine inhibits CYP2B6 and CYP2E1 (Taavitsainen et al. 2001; Zhang et al. 2001). Methoxsalen reduces first-pass metabolism of oral nicotine, decreases clearance of subcutaneously administered nicotine, and decreases urinary levels of 3′-hydroxycotinine in smokers (Sellers et al. 2000, 2003). Tranylcypromine has been shown to reduce first-pass metabolism of oral nicotine (Tyndale and Sellers 2001). As smokers smoke at least in part to maintain desired levels of nicotine in the brain, decreased metabolism and higher concentration of nicotine result in a reduction in the number of cigarettes smoked (Sellers et al. 2000). Also, as CYP2A6 is involved in the activation of carcinogenic NNK, inhibition of CYP2A6 routes the metabolism of NNK towards the inactive NNAL-glucuronide (Sellers et al. 2003). Thus, CYP2A6 inhibitors might be of use in reduction of smoking, thereby decreasing the exposure to carcinogenic metabolites, possibly reducing the risk of cancer, and enhancing the efficacy of nicotine replacement therapies.

6.3 Smoking

6.3.1 Inhibiting Effect of Smoking on Nicotine Clearance

Cigarette smoking itself influences the rate of metabolism of nicotine. Cigarette smoking is known to accelerate the metabolism of some drugs, especially the ones primarily metabolized by CYP1A2 (Zevin and Benowitz 1999). However, we found that the clearance of nicotine was significantly slower in cigarette smokers than in nonsmokers (Benowitz and Jacob 1993). In support of this observation are crossover studies comparing the clearance of nicotine in the same subjects when smoking compared to when not smoking. After 4 days of smoking abstinence, nicotine clearance was increased by 14% (Benowitz and Jacob 2000), and after 7 days of abstinence, nicotine clearance was 36% higher (Lee et al. 1987), when compared to overnight abstinence from cigarettes.

These studies suggest that there are substance(s) in tobacco smoke, as yet unidentified, that inhibit the metabolism of nicotine. Because nicotine and cotinine are metabolized by the same enzyme, the possibility that cotinine might be responsible for the slowed metabolism of nicotine in smokers was examined. In a study in which nonsmokers received an intravenous infusion of nicotine with and without pretreatment with high doses of cotinine, there was no effect of cotinine on the clearance of nicotine (Zevin et al. 1997). Also, carbon monoxide at levels and in patterns similar to those experienced during smoking had no effect on nicotine and cotinine clearance (Benowitz and Jacob 2000).

Recently, β-nicotyrine, a minor tobacco alkaloid, was shown to effectively inhibit CYP2A6 in vitro (Denton et al. 2004). Thus, β-nicotyrine is one candidate in the search for the inhibiting compound in tobacco smoke. Another possibility is that reduced nicotine clearance is due to downregulation of CYP2A6 expression, and not due to inhibition. Tyndale and coworkers have demonstrated that administration of nicotine for 21 days to monkeys in vivo decreases CYP2A6 activity (nicotine metabolism) by downregulating CYP2A6 mRNA and protein in liver (Schoedel et al. 2003). Interestingly, expression of both CYP2A and CYP3A5 mRNAs are markedly reduced in human pulmonary tissues in smokers compared to nonsmokers (Crawford et al. 1998; Hukkanen et al. 2003). The mechanisms of the downregulation are currently unknown.

6.3.2 Inducing Effect of Smoking on Glucuronidation

The excretion of 3′-hydroxycotinine *O*-glucuronide is induced by smoking, when compared to not smoking studied with a crossover design (Benowitz and Jacob 2000). The extent of nicotine and cotinine N-glucuronidation was not significantly affected by smoking. Smoking is known to induce glucuronidation of some drugs, such as propranolol and oxazepam (Liston et al. 2001). Urinary excretion of 3′-hydroxycotinine *O*-glucuronide is correlated with the excretion of

NNAL-*O*-glucuronide (Hecht et al. 1999b), which is formed by UGT1A9 and UGT2B7 (Ren et al. 2000). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), an AHR (arylhydrocarbon receptor) agonist, induces UGT1A9 but does not induce UGT2B7 in human Caco-2 cells (Munzel et al. 1999). Thus, UGT1A9 could be the inducible component of 3′-hydroxycotinine O-glucuronidation.

6.4 Racial and Ethnic Differences

Racial differences in nicotine and cotinine metabolism have been observed. We compared nicotine and cotinine metabolism in blacks and whites (Benowitz et al. 1999; Perez-Stable et al. 1998). The total and nonrenal clearance of cotinine was significantly lower in blacks than in whites (total clearance 0.57 vs. 0.76 ml min^{-1} kg^{-1}). Also, the fractional clearance of nicotine to cotinine, and the metabolic clearance of nicotine to cotinine were lower in blacks. The clearance of nicotine tended to be lower in blacks than in whites (18.1 vs. 20.5 ml min^{-1} kg^{-1}), but this difference was not significant. Excretion of nicotine and cotinine glucuronides was lower in blacks, while excretion of 3′-hydroxycotinine glucuronide was similar in both groups. Nicotine and cotinine glucuronidation appeared to be polymorphic in blacks, with evidence of slow and fast N-glucuronide formers. The distribution of glucuronidation was unimodal in whites. Polymorphic patterns of cotinine glucuronidation in blacks has been detected in other studies (de Leon et al. 2002). Slower metabolism of cotinine explains in part the higher cotinine levels per cigarette detected in blacks than in whites (Caraballo et al. 1998; English et al. 1994; Wagenknecht et al. 1990). One possible explanation for the slower cotinine metabolism in blacks is the significantly higher proportion of menthol cigarette smokers in blacks than in whites (69% vs. 22% in the general US population, 76% vs. 9% in our study) (Benowitz et al. 1999; Giovino et al. 2004). As discussed earlier, menthol cigarette smoking inhibits nicotine oxidation and glucuronidation (Benowitz et al. 2004).

Nicotine and cotinine metabolism among Chinese–Americans, Latinos, and whites has been compared (Benowitz et al. 2002b). Chinese–Americans had the lowest total and nonrenal clearance of nicotine and cotinine, and lowest metabolic clearance of nicotine via the cotinine pathway. Also, nicotine intake per cigarette was lower in Chinese–Americans than in Latinos and whites. No significant differences in nicotine and cotinine metabolism or nicotine intake were detected between Latinos and whites. Glucuronidation of nicotine and metabolites did not differ between the groups. Consistent with the findings in experimental studies, Kandel et al. found in an epidemiologic study that the 3HC/cotinine ratio in the urine of young adult smokers, reflecting CYP2A6 activity, was higher in whites and Hispanics than in blacks and Asians (Kandel et al. 2007).

7 Renal Excretion

Nicotine is excreted by glomerular filtration and tubular secretion, with variable reabsorption depending on urinary pH. With uncontrolled urine pH, renal clearance averages about 35–90 ml min^{-1}, accounting for the elimination of about 5% of total clearance. In acid urine, nicotine is mostly ionized and tubular reabsorption is minimized; renal clearance may be as high as 600 ml min^{-1} (urinary pH 4.4), depending on urinary flow rate (Benowitz and Jacob 1985). In alkaline urine, a larger fraction of nicotine is unionized, allowing net tubular reabsorption with a renal clearance as low as 17 ml min^{-1} (urine pH 7.0).

In vitro studies have shown that there are distinct transport systems for both basolateral and apical uptake of nicotine (Takami et al. 1998). Nicotine has been shown to be actively transported by kidney cells, most likely by the organic ion transporter OCT2 (Zevin et al. 1998; Urakami et al. 1998). Cimetidine decreases renal clearance of nicotine by 47% in nonsmoking volunteers (Bendayan et al. 1990). This is consistent with the inhibition of basolateral uptake by cimetidine detected in vitro. Mecamylamine reduces renal clearance of nicotine in smokers dosed with intravenous nicotine when urine is alkalinized, but not when urine is acidified (Zevin et al. 2000).

Renal clearance of cotinine is much less than the glomerular filtration rate (Benowitz et al. 2008b). Since cotinine is not appreciably protein bound, this indicates extensive tubular reabsorption. Renal clearance of cotinine can be enhanced by up to 50% with extreme urinary acidification. Cotinine excretion is less influenced by urinary pH than nicotine because it is less basic and, therefore, is primarily in the unionized form within the physiological pH range. As is the case for nicotine, the rate of excretion of cotinine is influenced by urinary flow rate. Renal excretion of cotinine is a minor route of elimination, averaging about 12% of total clearance. In contrast, 100% of nicotine N'-oxide and 63% of 3′-hydroxycotinine are excreted unchanged in the urine (Benowitz and Jacob 2001; Park et al. 1993).

The genetic contributions to nicotine and cotinine renal clearances have been estimated in a twin study (Benowitz et al. 2008b). This study found a substantial contribution of genetic factors to the net secretory/reabsorbtive clearances of nicotine and cotinine. These findings suggest either that the reabsorption of nicotine and cotinine are active processes and are influenced by the genetics of reabsorptive transporters, or that the active secretory component of renal clearance exerts a substantial effect on the clearance, even in the presence of net reabsorption. It is plausible that the genetic component of the variation in the reabsorbtive clearance of nicotine is determined by the corresponding variation in reabsorptive transporters.

As mentioned previously, renal failure markedly reduces total renal clearance, as well as metabolic clearance of nicotine and cotinine (Molander et al. 2000). Reduction of renal clearance is correlated with the severity of kidney failure; renal clearance is reduced by half in mild renal failure, and by 94% in severe renal impairment. Markedly elevated levels of serum nicotine have been detected in smoking patients with end-stage renal disease undergoing hemodialysis (Perry et al. 1984). This is explained not only by reduced renal clearance, but also by lower metabolic

clearance of nicotine in renal disease. It is speculated that accumulation of uremic toxins inhibits CYP2A6 activity or downregulates CYP2A6 expression in liver.

8 Nicotine and Cotinine Blood Levels During Tobacco Use and Nicotine Replacement Therapy

Blood or plasma nicotine concentrations sampled in the afternoon in smokers generally range from 10 to 50 ng ml^{-1}. Typical trough concentrations during daily smoking range between 10 and 37 ng ml^{-1} and typical peak concentrations range between 19 and 50 ng ml^{-1}. The increment in venous blood nicotine concentration after smoking a single cigarette varies from 5 to 30 ng ml^{-1}, depending on how a cigarette is smoked. In a recent study, the mean nicotine boost after smoking a cigarette was 10.9 ng ml^{-1} in smokers with no smoking abstinence on the study day (Patterson et al. 2003).

Blood levels peak at the end of smoking a cigarette and decline rapidly over the next 20 min due to tissue distribution. The distribution half-life averages about 8 min. Although the rate of rise of nicotine is slower for cigar smokers and users of snuff and chewing tobacco than for cigarette smokers, peak venous blood levels of nicotine are similar (Benowitz et al. 1988). Pipe smokers, particularly those who have previously smoked cigarettes, may have blood and urine levels of nicotine and cotinine as high as cigarette smokers (McCusker et al. 1982; Wald et al. 1981). Primary pipe smokers who have not previously smoked cigarettes tend to have lower nicotine levels. Likewise, cigar smokers who have previously smoked cigarettes may inhale more deeply and achieve higher blood levels of nicotine than primary cigar smokers, although on average, based on urinary cotinine levels, daily nicotine intake appears to be less for cigar smokers compared with cigarette or pipe smokers (Wald et al. 1984).

The plasma half-life of nicotine after intravenous infusion or cigarette smoking averages about 2 h. However, when half-life is determined using the time course of urinary excretion of nicotine, which is more sensitive in detecting lower levels of nicotine in the body, the terminal half-life averages 11 h (Jacob et al. 1999). The longer half-life detected at lower concentrations of nicotine is most likely a consequence of slow release of nicotine from body tissues. Based on a half-life of 2 h for nicotine, one would predict accumulation over 6–8 h (3–4 half-lives) of regular smoking and persistence of significant levels for 6–8 h after cessation of smoking. If a smoker smokes until bedtime, significant levels should persist all night. Studies of blood levels in regular cigarette smokers confirm these predictions (Benowitz et al. 1982b). Peak and trough levels follow each cigarette, but as the day progresses, trough levels rise and the influence of peak levels become less important. Thus, nicotine is not a drug to which smokers are exposed intermittently and which is eliminated rapidly from the body. On the contrary, smoking represents a multiple dosing situation with considerable accumulation while smoking and persistent levels for 24 h of each day.

Plasma levels of nicotine from nicotine replacement therapies tend to be in the range of low-level cigarette smokers. Thus, typical steady-state plasma nicotine concentrations with nicotine patches range from 10 to 20 ng ml^{-1}, and for nicotine gum, inhaler, sublingual tablet, and nasal spray from 5 to 15 ng ml^{-1} (Benowitz et al. 1987; Schneider et al. 2001). Usually ad libitum use of NRTs results in one-third to two-thirds the concentration of nicotine that is achieved by cigarette smoking (Schneider et al. 2001). However, users of 4-mg nicotine gum may sometimes reach or even exceed the nicotine levels associated with smoking (McNabb 1984; McNabb et al. 1982). For the sake of comparison, systemic doses from various nicotine delivery systems are as follows: cigarette smoking, 1–1.5 mg per cigarette (Benowitz and Jacob 1984; Jarvis et al. 2001); nicotine gum, 2 mg for a 4-mg gum (Benowitz et al. 1988); transdermal nicotine, 5–21 mg per day, depending on the patch; nicotine nasal spray, 0.7 mg per 1-mg dose of one spray in each nostril (Gourlay and Benowitz 1997; Johansson et al. 1991); nicotine inhaler, 2 mg for a 4-mg dose released from the 10-mg inhaler (Molander et al. 1996); nicotine lozenge, 1 mg for a 2-mg lozenge (Choi et al. 2003); oral snuff, 3.6 mg for 2.5 g held in the mouth for 30 min (Benowitz et al. 1988); and chewing tobacco, 4.5 mg for 7.9 g chewed for 30 min (Benowitz et al. 1988).

Cotinine is present in the blood of smokers in much higher concentrations than those of nicotine. Cotinine blood concentrations average about 250–300 ng ml^{-1} in groups of cigarette smokers. We have seen levels in tobacco users ranging up to 900 ng ml^{-1}. After stopping smoking, levels decline in a log linear fashion with an average half-life of about 16 h. The half-life of cotinine derived from nicotine is longer than the half-life of cotinine administered as cotinine (Zevin et al. 1997). This is caused by slow release of nicotine from tissues. Because of the long half-life there is much less fluctuation in cotinine concentrations throughout the day than in nicotine concentrations. As expected, there is a gradual rise in cotinine levels throughout the day, peaking at the end of smoking and persisting at high concentrations overnight. Cotinine levels produced by NRTs are usually 30–70% of the levels detected while smoking (Hurt et al. 1994; Schneider et al. 1995).

9 Biomarkers of Nicotine Exposure

Biomarkers are desirable for quantifying the systemic exposure of smokers to toxic constituents of smoke derived from tobacco use or from potential reduced harm products. Measures such as cigarettes per day are imprecise indicators of tobacco smoke exposure because of variability in how smokers smoke their cigarettes. There is considerable individual variability in smoke intake, even by people smoking the same brand of cigarettes (USDHHS 2001). Cigarette design and how the cigarette is smoked influence toxic exposures. For example, light cigarettes are smoked on average more intensely than are regular cigarettes. The optimal assessment of exposure to tobacco smoke would be the analysis of concentrations of chemicals of pathogenetic concern in body fluids of the exposed individual – termed a biological

marker or biomarker. A variety of biomarkers of tobacco smoke exposure have been proposed, as summarized in Table 2 and reviewed in detail previously (Hatsukami et al. 2003).

This section focuses on the use of nicotine and cotinine and other tobacco alkaloids as biomarkers of tobacco exposure. Other potential biomarkers of exposure to the particulate or gas phase of tobacco smoke are described in the review papers cited above.

Nicotine measurement is highly specific for tobacco use or exposure (in the absence of nicotine medication use), but because of nicotine's short half-life (2 h) the method is not recommended for general use. Cotinine is a highly specific and sensitive marker for tobacco use (in the absence nicotine medication use) and has the advantages of a fairly long half-life (16 h). When NRT is not being used, cotinine appears to be the best biomarker for tobacco use. When NRT is used, the minor tobacco alkaloids are useful biomarkers, as described below. A limitation of using cotinine is that it indicates ongoing exposure but not long-term exposure to tobacco smoke. Approaches to longer term monitoring include measurement of nicotine in hair or nails, as discussed below, or measurement of the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanol (NNAL) in urine, as described by (Hecht 2003).

9.1 Cotinine as a Biomarker for Intake of Nicotine

The presence of cotinine in biological fluids indicates exposure to nicotine. Because of the long half-life of cotinine it has been used as a biomarker for daily intake, both in cigarette smokers and in those exposed to secondhand tobacco smoke (Benowitz 1996). There is a high correlation among cotinine concentrations measured in plasma, saliva, and urine, and measurements in any one of these fluids can be used as a marker of nicotine intake. There is, however, individual variability in the quantitative relationship between steady state cotinine levels and intake of nicotine. This is because different people convert different percentages of nicotine to cotinine (usual range 50–90%), and because different people metabolize cotinine differently at different rates (usual clearance range 20–75 ml min^{-1}) (Benowitz 1996).

The relationship between nicotine intake and steady state cotinine blood levels can be expressed in the following way, based on steady state exposure conditions: $D_{nic} = CL_{COT} \times C_{COT} \div f$, where D_{nic} is the intake (dose) of nicotine, CL_{COT} is the clearance of cotinine, C_{COT} is the steady state blood concentration of cotinine and f is the fraction of nicotine converted to cotinine. On rearranging the equation, $D_{nic} = (CL_{COT} \div f) \times C_{COT} = K \times C_{COT}$ where K is a constant that converts a given blood level of cotinine to nicotine intake. On average, $K = 0.08\,mg\ 24\,h^{-1}\ ng^{-1}\ ml^{-1}$ (range 0.05–1.1, CV = 21.9%). Thus, a cotinine level of 30 ng ml^{-1} in blood corresponds on average to a nicotine intake of 24 mg per day.

Table 2 Biomarkers of tobacco exposure

Biomarker	Precursor	Specimen	$t_{1/2}$	Tobacco specific	Other sources
Nicotine*	Nicotine	Blood, urine, saliva, hair	1–2 h	Yes	Nicotine replacement products
Cotinine*	Nicotine	Blood, urine, saliva, hair	16–18 h	Yes	Nicotine replacement products
Anatabine*	Anatabine	Urine	10–16 h	Yes	None
NNAL, NNAL-glucuronides	NNK (TSNA)	Blood, urine	6 week	Yes	None
Exhaled CO	Carbon monoxide	Exhaled air	2–6 h	No	Traffic, body formation
Carboxyhemoglobin	carbon monoxide	Blood	4–6 h	No	Traffic, body formation
1-Hydroxypyrene and other polycyclic aromatic hydrocarbon (PAH) metabolites	PAHs	Urine	20 h	No	Traffic, grilled meat, occupation, biomass combustion in homes
Mercopturic acid metabolites	1,3-Butadiene	Urine	–	No	Traffic, combustion products
Mercopturic acid metabolites	Acrolein	Urine	–	No	Traffic, combustion products
Acetonitrile	Acetonitrile	Urine, blood, exhaled air	32 h	No	None
S-Phenyl-mercapturic acid	Benzene	Urine	9 h	No	Traffic, combustion products
Thiocyanate	Hydrogen cyanide	Serum, saliva, urine	7–14 days	No	Diet

NNAL 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; *NNAL-gluc* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide; *NNK* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; *TSNA* tobacco-specific nitrosamines; *PAH* polycylcic aromatic hydrocarbons

In studies of smoking cessation, anatabine is recommended as nicotine replacement therapies will lead to the presence of nicotine and cotinine without any tobacco exposure

While cotinine functions fairly well as a marker of nicotine intake, it is not perfect due to individual variation in metabolism as discussed previously. As described earlier in this chapter, cotinine metabolism is affected by factors such as race, gender, age, genetic variation in the liver enzyme CYP2A6, and/or by the presence of pregnancy, liver or kidney disease. Another limitation to the use of cotinine is that, given an average half-life of 16 h, cotinine levels reflect relatively short-term exposure to tobacco (that is, over the past 3–4 days).

9.2 Nicotine and Cotinine in Hair and Nails

The use of hair as a material in which to measure nicotine and cotinine has been proposed as a way to assess long-term exposure to nicotine from tobacco products. Nicotine and cotinine are incorporated into hair as it grows over time. The average rate of hair growth is 1 cm per month. Thus, measurements of levels of nicotine may provide a way of assessing exposure of a person to nicotine over several months (Al-Delaimy et al. 2002; Florescu et al. 2007).

Potentials problems with the use of hair include a strong influence of hair pigmentation on nicotine and cotinine binding and uptake (Dehn et al. 2001). Nicotine and cotinine are bound to melanin. As a result, dark hair binds much more nicotine than does blond or white hair. This makes comparison across individuals difficult. Also, hair is exposed to nicotine and cotinine from sweat and from sebaceous gland secretions, and to nicotine from environmental tobacco smoke exposure. Washing the hair before analysis may reduce this problem of environmental contamination, but it is not likely to remove all environmental nicotine and cotinine.

Nicotine and cotinine, as well as NNAL, can be measured in nail clippings (Stepanov et al. 2007). Toenail clippings are easy to collect and store and represent cumulative exposure as nails grow at a rate of about 0.1 cm per month. In a group of smokers, the average toenail biomarker concentrations were 5.4 ng nicotine and 0.67 ng cotinine per mg toenail. Plasma levels of nicotine and cotinine were significantly but moderately correlated with toenail levels. Thus, hair or toenail measurements of nicotine or cotinine (or NNAL) are promising biomarkers of long-term tobacco exposure.

9.3 Dietary Sources

Dietary sources of nicotine have been alleged to be a potential confounder of cotinine levels used in measurement of secondhand smoke exposure. Several foods contain small amounts of nicotine (Siegmund et al. 1999). However, the levels of nicotine in foods are quite low. Based on nicotine levels in foods and the usual daily consumption of various nicotine-containing foods, it has been determined that

the levels of cotinine produced by even a diet high in nicotine-containing foods is lower than that seen in individuals exposed to moderate levels of secondhand smoke (Benowitz 1996).

9.4 Minor Tobacco Alkaloids

The primary alkaloid in tobacco is nicotine, but tobacco also contains small amounts of minor alkaloids such as anabasine, anatabine, myosmine, and others. The minor alkaloids are absorbed systemically and can be measured in the urine of smokers and users of smokeless tobacco (Jacob et al. 1999). The measurement of minor alkaloids is a way to quantitate tobacco use when a person is also taking in pure nicotine from a nicotine medication or a nontobacco nicotine delivery system. This method has been used to assess tobacco abstinence in clinical trials of smoking cessation with treatment by nicotine medications (Jacob et al. 2002).

9.5 Optimal Cotinine Cut-Points to Distinguish Tobacco Use From No Tobacco Use

Based on the work of Jarvis and coworkers, who measured cotinine levels in individuals attending outpatient clinics in the United Kingdom in the early 1980s, an optimal plasma or saliva cotinine cut-point of 15 ng ml^{-1} or a urine cotinine of 50 ng ml^{-1} were determined to discriminate smokers from nonsmokers (some of whom are exposed to secondhand smoke) (Benowitz et al. 2002a). The optimal cut-point depends on the smoking behavior of the smokers and the magnitude of exposure to secondhand smoke. Data from the National Health and Nutrition Examination Surveys (NHANES) from 1999 to 2004 were recently analyzed to assess the optimal serum cotinine in the US population at present (Benowitz et al. 2008a). Using receiver operator characteristic curve analysis, the optimal cotinine cut-points were 3.08 ng ml^{-1} for adults (sensitivity 96.3%, specificity 97.4%) and 2.99 ng ml^{-1} for adolescents (sensitivity 86.5%, specificity 93.1%). The decline in the optimal cut-point since 1980 is likely due to the marked reduction in secondhand smoke exposure in the general US population. Of note is that the cut-points are much lower for Mexican Americans than for whites or African Americans, most likely due to both more occasional smoking and lower exposure to secondhand smoke.

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Nicotine Content and Delivery Across Tobacco Products

Mirjana V. Djordjevic and Kelly A. Doran

Contents

Abstract Nicotine is the principal alkaloid in both commercial and homemade products (e.g., cigarettes, smokeless tobacco, bidis, waterpipes) followed by nornicotine, anabasine, anatabine, and many other basic substances that contain a cyclic nitrogenous nucleus. Tobacco types, leaf position on the plant, agricultural practices, fertilizer treatment, and degree of ripening are among some prominent factors that determine the levels of alkaloids in tobacco leaf. From a random examination of 152 cultivated varieties of *Nicotiana tabacum*, a range of alkaloid variation between 0.17 and 4.93% was determined. In fact, every step in tobacco production that affects plant metabolism will influence the level of alkaloid content to a certain degree.

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Depending on blending recipe, type and amount of additives, and product design, all types of tobacco products contain a very wide range of nicotine concentration. However, the ultimate emission of nicotine to the user, exposure, and psychopharmacological effects depend not only on the content and emission, but also on the relationship between the product and the user.

1 Introduction

Tobacco use is primarily due to psychopharmacological effects of nicotine (Henningfield et al. 2006). Nicotine is a tobacco alkaloid, a basic substance that contains a cyclic nitrogenous nucleus. In *Nicotiana* plants, most alkaloids are 3-pyridyl derivatives[1]. In cured leaf of Maryland Robinson Medium Broadleaf, 24 pyridine derivatives were identified, including nicotine, nornicotine, anabasine, oxynicotine, myosmine, 3-acetylpyridine, 2, 3′-dipyridyl, nicotinamide, anatabine, nicotinic acid, and unidentified pyridine alkaloids of derivatives thereof (Tso 1990). Nicotine is the principal alkaloid in commercial tobacco (this was confirmed in 34 out of 65 *Nicotiana* species); nornicotine, rather than nicotine, appears to be the main alkaloid in 19 out of 65 species; and anabasine is the third most important. In addition to the above-mentioned principal and minor alkaloids, the presence of many trace amounts of new alkaloids or their derivatives were frequently reported, including, for example, 2.4′-dipyridyl, 4, 4′-dipyridyl, *N*′-formylanabasine, *N*′-formylanatabine, *N*′-acetylanatabine, *N*′-hexanoyl-nornicotine, *N*′-octanoyl-nornicotine, 1′-(6-hydroxyoctanoyl) nornicotine, and 1′-(7-hydroxyoctanoyl) nornicotine.

Commercial tobacco, or *Nicotiana tabacum* (*N. tabacum*), is one of the more than 64 established species in the genus *Nicotiana*. Among those species, 45 are indigenous to North or South America, and 15 to Australia. Of the many American species, *N. tabacum* is the only one grown commercially in the USA at the present time (Tso 1990). In Russia and some of the Asiatic countries, *N. rustica* is also grown more or less extensively, though chiefly for local consumption. All 64 of the *Nicotiana* species tested by Sisson and Severson (1990) contained a measurable alkaloid fraction (at least 10 $\mu g\ g^{-1}$). There was a wide range in total alkaloid levels, with a 400-fold difference among field-grown species.

Tobacco types, leaf position on the plant, agricultural practices, fertilizer treatment, and degree of ripening are among some prominent factors that determine the levels of alkaloids in *Nicotiana* plants. In fact, every step in tobacco production that affects plant metabolism will influence the level of alkaloid content to a certain degree (Tso 1990). The Maryland and Turkish types of tobacco are generally low in nicotine; the flue-cured, burley, Cuban, and Connecticut cigar wrappers are medium; and the Pennsylvania, dark fire-cured tobaccos, especially *N. rustica*, are high in nicotine content. Under favorable conditions (e.g., fertile soils under irrigation over

[1] Indole alkaloids, such as harmane and norharmane, were also reported to be present in tobacco but in minute quantities.

a period of years), *N. rustica* consistently produced more nicotine than *N. tabacum* (Bhide et al. 1987; Sisson and Severson 1990). From a random examination of 152 cultivated varieties of *N. tabacum*, a range of alkaloid variation between 0.17 and 4.93% was found (Tso 1990).

Tobacco leaves have the highest content of nicotine, roots have less, and stalks have the least. Alkaloid level increases as plants mature, especially during the period after topping (Burton et al. 1983, 1989b, 1994; Djordjevic et al. 1989; Peele et al. 1995; Walton et al. 1995; DeRoton et al. 2005). Marked increase of nicotine was generally accomplished with the increased rate of nitrogen fertilization (Chamberlain and Chortyk 1992). In lamina of air-cured and flue-cured tobacco, nicotine content increased from 41.82 to 65.77 mg g^{-1} tobacco and 30.66 to 33.51 mg g^{-1}, respectively, when nitrogen was applied from 0–300 lbs $acre^{-1}$; in midribs of air-cured and flue-cured tobacco, nicotine content increased from 2.74 to 6.5 mg g^{-1} tobacco and 6.46 to 6.78 mg g^{-1}, respectively.

2 Nicotine Content in Cured Tobacco Leaves

Nicotine content varies considerably in different tobacco types (e.g., sun-cured oriental, flue-cured Virginia, air-cured burley, and air-cured dark tobacco; Table 1). Oriental tobacco commonly used for manufacturing cigarettes in the former USSR contained 1.8–12.6 mg nicotine per gram of dry tobacco (Djordjevic et al. 1991). Nicotine content in flue-cured laminae from the third priming (leaves from the upper stalk position) of NC alkaloid isolines (Virginia bright tobacco) contained 6.52–60.4 mg nicotine per gram of dry tobacco (Djordjevic et al. 1989). Burley tobacco lamina contained 35.6–47.73 mg nicotine per gram dry tobacco (Burton et al. 1989a; MacKown et al. 1988). Nornicotine and anatabine concentrations also show wide range of concentrations in different tobacco types. The concentrations of nicotine, nornicotine, and anatabine in Burley midribs were significantly lower than in laminae: 5.5–19.48, 0.33–0.51, and 0.15–0.45 mg, respectively. As reported by MacKown et al. 1988, reconstituted tobacco sheets contained 5.1 mg nicotine per gram, 0.2 mg nornicotine, and 0.1 mg anatabine.

Tobacco leaves harvested from the bottom of Virginia tobacco plants contained the lowest amount of nicotine, whereas the leaves from the top contained the highest amount (37.37 and 60.4 mg g^{-1} dry tobacco, respectively) (Djordjevic et al. 1989).

Table 1 Alkaloid content in lamina of different tobacco types[a]

Alkaloid	Alkaloid content (mg g^{-1})		
	Oriental tobacco	Virginia tobacco	Burley tobacco
Nicotine	1.80–12.6	6.52–60.4	35.6–47.73
Nornicotine	0.05–1.32	0.14–6.47	0.9–2.09
Anatabine	0.02–1.60	0.14–2.17	0.9–2.31

[a]Djordjevic et al. 1991, 1989; Burton et al. 1989a; MacKown et al. 1988

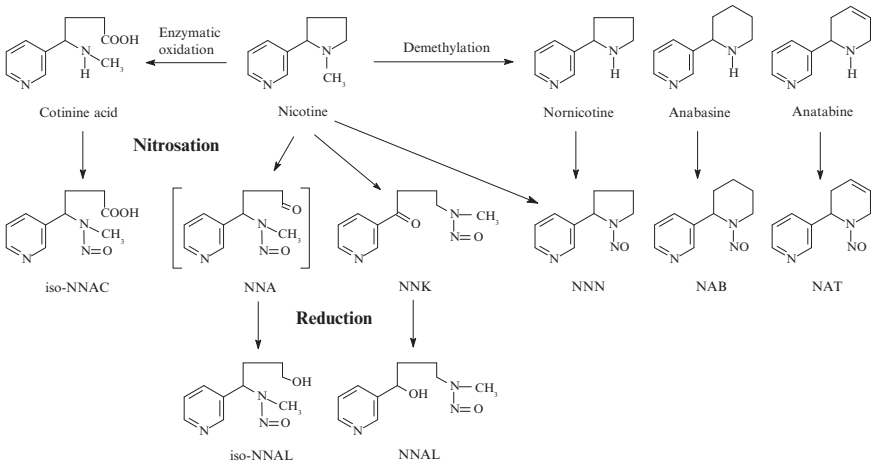

Fig. 1 Formation of tobacco-specific *N′*-nitrosamines (Hoffmann et al. 1995). *iso-NNAC*, 4-(methylnitrosoamino)-4-(3-pyridyl)butyric acid; *iso-NNAL*, 4-(methylnitrosoamino)-4-(3-pyridyl)-1-butanol; *NAB*, *N′*-nitrosoanabasine; *NAT*, *N′*-nitrosoanatabine; *NNA*, 4-(methylnitrosoamino)-4-(3-pyridyl)butanal; *NNAL*, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol; *NNK*, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; *NNN*, *N′*-nitrosonornicotine (Note: *NNA* is a very reactive aldehydes and has therefore never been quantified in tobacco or tobacco smoke)

The concentration of alkaloids was reported to be the lowest at the base and the tip of the leaf, and greatest at the periphery of the leaf. Thus, nicotine content in the lamina of a dark air-cured tobacco (Ky 171) varies from 33.06 to 76.10 mg g^{-1}; nornicotine from 0.37 to 0.76 mg g^{-1}; and anatabine from 0.41 to 0.82 mg g^{-1} (Burton et al. 1992).

Both nicotine and nornicotine give rise to tobacco-specific *N*-nitrosamines (TSNA; Fig. 1) during all stages of tobacco production, from growing in the field to curing, processing, and storage, as well as during product manufacturing and through combustion during puffing of combustible products (Hoffmann et al. 1994). TSNA are present in both smoked and nonsmoked tobacco products, and their concentrations vary dramatically from one product type to another, from one brand of product to another, and from one country to another (IARC 2004, 2007). Upon the evaluation of scientific evidence regarding carcinogenic risks to humans, the International Agency for Research on Cancer (IARC) designated nicotine-derived 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and *N′*-nitrosonornicotine (NNN) as *carcinogenic to humans (Group 1)* (IARC 2007).

During the past two decades, it has been demonstrated that there are available technologies, primarily curing practices, to control the formation of carcinogenic TSNA and their precursors in tobacco, thus enabling approaches for lowering of NNN and NNK levels in tobacco products (Burns et al. 2008; O'Connor and Hurley 2008). Genetically, flue-cured and dark tobaccos have fairly low levels of nornicotine and the trait is stable, while burley tobaccos have higher levels and tend

to be highly variable. Nornicotine accumulation in burley tobacco is of concern, because burley is one of the major constituents of American blend cigarettes and nornicotine is the major precursor of carcinogenic NNN (Hoffmann et al. 2001). As a result of screening burley lines, low converter varieties (plants that convert or demethylate nicotine to nornicotine) have been released, and this has greatly reduced the level of nornicotine (and hence NNN) in the national burley crop (Jack et al. 2007; Siminszky et al. 2007). Similarly to reduction of NNN, NNK, and nornicotine, new technologies and approaches should be utilized to reduce nicotine levels in tobacco to curb both the addiction to tobacco products and their toxicity (Benowitz and Henningfield 1994; Henningfield et al. 1998; Benowitz et al. 2007). To that end, several cigarette brands with a very low content of nicotine in tobacco were introduced on the market: the brand Next (Philip Morris USA) containing 0.03 mg nicotine per gram of dry tobacco (the tobacco blend was denicotinized by the supercritical fluid extraction method) (Djordjevic et al. 1990), and three versions of Quest (Vector Tobacco Inc.) containing 0.6, 0.3, and <0.05 mg nicotine, respectively (the cigarette blend was made using genetically modified tobacco) (Chen et al. 2008; Strasser et al. 2007).

Given the wide range of nicotine content in various tobacco types or in leaves harvested from different plant positions, and also considering the country of origin and variability from year-to-year crops due to climate conditions and agricultural practices, manufacturers have unparallelled opportunity to manipulate the nicotine content in cigarettes and nicotine delivery to the smoker, chiefly by blending tobaccos. Additionally, a variety of product design strategies and application of additives such as ammonia or ammonia-derived agents play important roles in nicotine bioavailability, as well as in physiological and addictive effects (Henningfield et al., 2004).

3 Nicotine Content in Factory-Made Cigarettes

Until the last two decades, only flue-cured tobaccos were used in cigarettes in the UK and Finland, and they were the predominant type used in Canada, Japan, China, Australia, and New Zealand. Air-cured tobaccos were preferred in France, southern Italy, some parts of Switzerland and Germany, and South America; cigarettes made exclusively from sun-dried tobaccos are popular in Greece and Turkey. In the rest of Western Europe and in the USA, cigarettes contain blends of flue-cured and air-cured tobaccos as major components (Hoffmann et al. 2001). Today, in many countries all over the world, including the UK and France, American blend cigarettes are gaining market shares. Since the early 1990s, the typical composition of an American blended cigarette was 35% flue-cured (Virginia) tobaccos, 30% air-cured (burley) tobaccos, and a few percent of Maryland and oriental tobaccos, as well as reconstituted tobacco sheets.

3.1 Nicotine Content in Cigarette Filler

The nicotine content in tobacco from cigarettes sold worldwide shows a wide variation (IARC 2004). Counts and coauthors reported on the nicotine content in the tobacco filler of 48 Philip Morris USA and Philip Morris International commercial filtered cigarettes from numerous international market regions (Counts et al. 2005). The majority contained blends of bright flue-cured (Virginia), burley air-cured, and sun-cured oriental tobaccos, with inclusions of expanded tobaccos, processed tobacco, or processed stems. Four cigarettes contained primarily bright tobaccos. Nine brands contained carbon (also known as "charcoal") in their filter construction.

The nicotine concentrations in Philip Morris' sample of international brands ranged from 13.79 to 23.18 mg g^{-1} of dry tobacco, and ammonia concentrations from 0.16 to 3.51 mg g^{-1} of tobacco. Data presented in Table 2 show that cigarettes that ranked as very low-yield ($\leqslant$4.9 mg tar per cigarette), low-yield (5–9.9 mg tar), and moderate-yield (10–14.9 mg tar), based on tar and nicotine deliveries measured by the Federal Trade Commission/International Standards Organization (FTC/ISO) machine-smoking method (IARC 1986), contained similar amounts of nicotine and ammonia in tobacco filler. However, it is notable that very low-yield cigarettes tend to contain somewhat higher amounts of both nicotine and ammonia in the tobacco column, both in American and Virginia blend cigarettes. The latter is a very important observation, suggesting that even smokers of very low-yield cigarettes can extract the desirable nicotine dose by adopting specific smoking behaviors, regardless of the ranking based on the standard machine-smoking method. As for the brands with charcoal in the filter tip, there is no apparent difference in tobacco nicotine content compared to American blend cigarettes, which are manufactured with filter tips made exclusively of cellulose acetate fibers.

Table 2 Content of nicotine and ammonia in tobacco filler of cigarettes ($n = 48$) with different smoke yields as determined by standard FTC/ISO machine-smoking method (Counts et al. 2005)

Cigarette	Nicotine content (mg g^{-1} dry tobacco)	Ammonia content (mg g^{-1} dry tobacco)
American blend (44 brands)		
Moderate yield[a]	13.70–20.12	0.85–3.32
Low yield[b]	13.79–19.48	0.84–3.51
Very low yield[c]	16.26–23.18	1.27–3.28
Virginia blend (4 brands)		
Moderate yield[a]	16.91–17.11	0.18–0.45
Low yield[b]	15.36	0.46
Very low yield[c]	18.64	0.24
Charcoal filter (9 brands)		
Moderate yield[a]	15.8–18.82	1.17–2.83
Low yield[b]	14.42–19.17	0.84–3.25
Very low yield[c]	20.24	1.54

Yield ranking of cigarettes according to IARC (1986)
[a] 10–14.9 mg FTC tar
[b] 5–9.9 mg FTC tar
[c] $\leqslant$4.9 mg FTC tar

Table 3 Content of nicotine in tobacco filler of cigarettes with different smoke yields as determined by standard FTC/ISO machine-smoking method (Kozlowski et al. 1998)

Country of origin of cigarettes	Nicotine content (mg g^{-1} tobacco)			
	High[a]	Moderate[b]	Low[c]	Very low[d]
United States, $n=32$	9.5–13.4	8.9–11.4	7.2–11.5	8.7–10.9
Canada, $n=23$	8.0–15.4	11.6–18.3	11.9–16.7	11.2–14.4
United Kingdom, $n=37$	NR	9.0–17.5	9.9–14.3	10.7–15.7

Yield ranking of cigarettes according to IARC (1986)
[a] >15 mg tar
[b] 10−14.9 mg tar
[c] 5−9.9 mg tar
[d] ⩽4.9 mg FTC tar
n number of brands tested
NR not reported

Cigarette characteristics that influence nicotine delivery to the smoker (including nicotine and ammonium content in tobacco filler) as well as human smoking behavior, deserve special consideration, because nicotine causes and maintains addiction that leads to chronic exposure to a chemical toxicant with known harmful health effects, including cancer (UDHHS 2004; IARC 2004; 2007).

Kozlowski and coauthors reported on the nicotine content in 92 brands of cigarettes (32 American, 23 Canadian, and 37 British) (Kozlowski et al. 1998). The total nicotine content of tobacco averaged 10.2 mg g^{-1} tobacco (7.2–13.4 range) in the USA, 13.5 mg g^{-1} (8.0–18.3 range) in Canada, and 12.5 mg g^{-1} (9–17–5 range) in the UK. It is apparent, from the data presented in Table 3, that there is no difference in the nicotine content of tobacco, regardless of the type of cigarettes. In summary, the similar nicotine content in the filler of cigarettes with a wide range of FTC machine-smoke yields, as shown by Djordjevic et al. (1990), Kozlowski et al. (1998), and Counts et al. (2005), clearly confirms that the elasticity was built into the design of the cigarettes, so that smokers can extract as much nicotine as they needed by changing puffing topography.

Stepanov and coauthors compared the nicotine content in tobacco from cigarettes produced in the USA, in Moldavia, and in foreign cigarettes commercialized in Moldavia (Stepanov et al. 2002). They reported similar levels of nicotine in domestic Moldavian cigarettes (9.6–19.6 mg nicotine per gram wet weight), imported brands (13.5–15.1 mg nicotine per gram wet weight), and cigarettes consumed in the USA (17.6–19.5 mg nicotine per gram wet weight).

3.2 Nicotine Content in Cigarette Smoke (Machine-Smoking Methods)

Traditionally, smoke yields expressed per cigarette have been measured by the machine-smoking method that was implemented by the Federal Trade Commission

(FTC) in 1967, based on the protocol developed by the American Tobacco Company in the 1930s (Bradford et al. 1936; Pillsbury 1996). Internationally, this method is also known as the International Standards Organization (ISO) method. Mainstream smoke tar, nicotine, and carbon monoxide (CO) are determined when cigarettes are smoked by machine with a puff volume of 35 cm^3; puffs are taken once every 60 s and the duration of the puff is 2 s. Cigarettes are smoked to a prescribed final butt length, and are tested without blocking of ventilation holes in the cigarette filter. Based on the FTC report, tar and nicotine delivery in US cigarettes, weighted by sales, declined from 21.6 and 1.35 mg per cigarette, respectively, in 1967 to 12.0 and 0.88 mg per cigarette, respectively, in 1998 (FTC 2000).

Calafat and coauthors conducted a survey of nicotine, tar, and CO deliveries from 77 cigarette brands purchased in 35 countries from the six WHO regions, using the FTC/ISO machine-smoking methods (Calafat et al. 2004). Mainstream smoke nicotine deliveries varied from 0.5–1.6 mg per cigarette. Analysis of the smoke deliveries suggested that cigarettes from the Eastern Mediterranean, Southeast Asian, and Western Pacific WHO regions tended to have higher tar, nicotine, and CO smoke deliveries than brands from the European, American, or African WHO regions surveyed.

The FTC/ISO yields of nicotine in 25 commercial UK cigarettes made from bright tobaccos ranged from 0.11–0.94 mg per cigarette (Gregg et al. 2004). These data reflect the compliance with the directive of the European Parliament, which mandated that "from January 1, 2004, the yields of cigarettes released for free circulation, marketed or manufactured in the Member states shall not be greater than 10 mg per cigarette for tar, 1 mg per cigarette for nicotine, 10 mg per cigarette for carbon monoxide." (European Parliament 2001). International comparison of the ranges of mainstream smoke nicotine yields showed a wide variation (0.1–2.7 mg per cigarette), with the highest emissions measured in cigarettes from France, Thailand, the UK, and the USA (IARC 2004).

Currently, there is scientific consensus that FTC/ISO per cigarette smoke yields do not provide valid estimates of human exposure, or of relative human exposure, when smoking different brands of cigarettes (National Cancer Institute 2001; Stratton et al. 2001; Burns et al. 2008). Machine smoking regimens other than that of the FTC/ISO have also been examined, particularly ones with more intense puffing parameters and those that partially or completely block the ventilation holes in cigarette filters. The examples include those developed by the state of Massachusetts and the Canadian Government. The Massachusetts method prescribes drawing 45-cm^3 puffs once every 30 s, the duration of each puff is 2 s, and 50% of filter ventilation holes are blocked during smoking; the Health Canada method prescribes drawing 55-cm^3 puffs once every 30 s, the duration of each puff is 2 s, and 100% of filter ventilation holes are blocked during smoking (Borgerding and Klus 2005). These two regimens generally produce higher yields per cigarette (Table 4; Counts et al. 2005), and reduce differences between brands in the yields. Nevertheless, these regimens continue to maintain a ranking of brands by tar and nicotine yield per cigarette. Also, the rankings by yield per cigarette using these more intense regimens do not provide valid estimates of human exposure, or of the relative exposure, ex-

Table 4 Nicotine yields in the mainstream smoke of Philip Morris cigarettes ($n = 48$) generated under different smoking conditions (Counts et al. 2005)

Method	Nicotine yield (mg per cigarette)		
	Moderate[a]	Low[b]	Very low[c]
FTC	0.67–1.04	0.44–0.77	0.10–0.46
Masachusetts	1.65–2.17	1.07–1.70	0.51–1.20
Health Canada	1.48–2.56	1.43–2.17	1.07–1.85

Yield ranking of cigarettes according to IARC (1986)
[a] 10−14.9 mg tar
[b] 5−9.9 mg tar
[c] ⩽4.9 mg FTC tar

perienced by smokers when they smoke different brands of cigarettes (Burns et al. 2008). Normalization of the machine-generated yields per mg nicotine, or per mg tar, does not eliminate the variation in the values measured by the different smoking regimens. For example, the differences in the yields of smoke toxicants per mg nicotine (e.g., NNK, NNN, acetaldehyde, acrolein, benzene, benzo(a)pyrene, 1,3-butadiuene, carbon monoxide), as recommended for regulation by the WHO Study Group on Tobacco Regulation (TobReg) with these different regimens, likely reflect differences in temperature of combustion, rates of air flow at the point of combustion, and other factors that result from the differences in puff profiles used (Burns et al. 2008).

The fate of nicotine in burning full-flavor cigarettes is affected by the manner in which the cigarette is smoked. The greater percentage of labeled nicotine in the tobacco column remains intact during the smoking process as smoking intensity increases (Yu et al. 2006). As smoking regimen intensity increased, the amount of nicotine pyrolisis and oxidation products detected in sidestream smoke decreased, while marginal increases in these compounds were observed in mainstream smoke and in the cigarette butt.

Connolly and coauthors undertook the study to find out whether nicotine yields in the smoke of cigarettes sold in the USA, as measured by the Massachusetts machine-smoking method, would show an overall increase over time or an increasing trend limited to any particular market category (e.g., full-flavor versus light, medium/mild, or ultra-light; mentholated versus nonmentholated), manufacturer, or brand family or brand style, and whether nicotine yields in smoke would be associated with measurable trends in cigarette design (Connolly et al. 2007). They reported a statistically significant trend in increased nicotine yield of 0.019 mg (1.1%) per cigarette per year over the period of 1997–2005, and 0.029 mg (1.6%) per cigarette per year over the period 1998–2005. The increasing trend was observed in all major market categories. Nicotine yield in smoke was positively associated with nicotine concentration in the tobacco and the number of puffs per cigarette, both of which showed increasing trends during the study period.

3.3 Nicotine in Cigarette Smoke (Human Smoking Patterns)

A single machine testing regimen produces a single set of toxicant yields. In contrast to the machine, individual smokers vary their puffing patterns when smoking different cigarettes of the same brand (including blocking filter ventilation holes and smoking to a certain butt length), and cigarette design changes can lead smokers to systematically change how they puff cigarettes. Thus, even yields using the more intense Massachusetts and Health Canada machine-smoking regimens have the potential to mislead smokers when expressed per cigarette. Thus, the machine-measured yields should not be used to support claims of reduced exposure or risk (Burns et al. 2008).

Compared with the FTC/ISO protocol values, 56 smokers of low-yield brands and 77 smokers of medium-yield brands took in statistically significantly larger puffs (48.6 and 44.1 mL, respectively) at statistically significantly shorter intervals (21.3 and 18.5 s, respectively) (Djordjevic et al. 2000). Thus, they received, respectively, 2.5 and 2.2 times more nicotine and 2.6 and 1.9 times more tar than FTC-derived amounts, as well as about twofold higher levels of the nicotine-derived carcinogen NNK. Smokers of low-yield cigarettes received 1.74 mg (1.54–1.98) nicotine from their cigarette, whereas smokers of medium-yield cigarettes received 2.39 mg (2.2–2.6) nicotine. Delivery of NNK among smokers of low-yield cigarettes was 112.9 ng (158.3–219.7) per cigarette, and was 250.9 ng (222.7–282.7) per cigarette among smokers of medium-yield cigarettes. Although there was a slight difference in "at the mouth" delivery as shown by Djordjevic et al. 2000, the exposure to smoke toxicants, as measured by urinary biomarkers, revealed similar uptake of nicotine and the lung carcinogen NNK by smokers of regular, light, and ultra light cigarettes (Hecht et al. 2005). Levels of urinary metabolites expressed per unit of delivered parent compounds, measured in the mainstream smoke generated by mimicking human smoking patterns, decreased with increased smoke emissions (Melikian et al. 2007a). In smokers of low-, medium-, and high-yield cigarettes, the respective cotinine (ng mg^{-1} creatinine)-to-nicotine (mg day^{-1}) ratios were 89.4, 77.8, and 57.1 (low versus high; $p = 0.06$); the 4-(methylnitrosoamino)-1-(3-pyridil)-1-butanol (NNAL) (pmol mg^{-1} creatinine)-to-NNK (ng day^{-1}) ratios were 0.81, 055, and 0.57 (low versus high; $p = 0.05$). Similarly, means of cotinine per unit of delivered nicotine in smokers who consumed <20 cigarettes per day was 3.5-fold higher than in those who smoked >20 cigarettes per day. Likewise, a negative correlation was observed between cotinine–nicotine ratios and delivered doses of nicotine in subgroups of smokers who used the identical brand of cigarette, namely filter tip-ventilated Marlboro ($r = -0.59$), which is popular brand among European Americans, and Newport ($r = -0.37$), a menthol-flavored cigarette without filter tip vents, which is preferred by African Americans. Thus, intensity of the exposure significantly affects the levels of urinary biomarkers of exposure, and this inverse relationship phenomenon should be further explored.

Melikian and coworkers also reported on gender differences in delivered nicotine dosages as a result of specific puffing behaviors (Melikian et al. 2007b). The geometric means of emissions of nicotine from cigarettes were 1.92 mg per cigarette

(95% CI = 1.8–2.05) for women versus 2.2 mg per cigarette (95% CI = 2.04–2.37) for men ($p = 0.005$). Similarly, cigarettes smoked by women yielded 139.5 ng per cigarette of the carcinogenic NNK (95% CI = 128.8–151.0), compared with 170.3 ng per cigarette (95% CI = 156.3–185.6) for men ($p = 0.0007$). The gender differences with regard to cigarette smoke yields of toxicants were more profound in European Americans than in African Americans. On average, African American men's smoking behavior produced the highest emissions of select toxicants from cigarettes, and European American female smokers had the lowest exposure to nicotine and carcinogens.

4 Nicotine Content in Other Combustible Tobacco Products

4.1 Roll-Your-Own Cigarettes

Although factory-made (FM) cigarettes dominate the world market, the use of roll-your-own (RYO) cigarettes has increased substantially in Thailand (58%), New Zealand (32%), the UK (28.4%), Australia (24.2%), Malaysia (17%), Canada (17.1%), the USA (6.7%), and in some European countries such as Norway (15.5%) (Young et al. 2006, 2008; Laugesen 2003; Wangan and Biørn 2001). Most RYO smokers choose to make their own cigarettes because they are less expensive than FM products, or because they perceive RYO as less harmful. According to Young and coauthors, the use of RYO cigarettes was associated with having lower annual income, male sex, younger average age, higher level of nicotine addiction, and more positive perception of tobacco use (Young et al. 2006).

Based on the FTC/ISO machine-smoking method, the nicotine yields reported for the five brands of fine-cut tobaccos used in preparation of RYO cigarettes were 1.5–1.8 mg per unit, whereas the nicotine delivery from 35 commercial cigarettes was lower, at 0.09–1.4 mg per cigarette (Kaiserman and Rickeryt 1992). In addition, the levels of the lung carcinogen benzo[a]pyrene (BaP) in the smoke of RYO cigarettes were between 22.9 and 26.3 ng per unit, compared to 3.36–28.39 ng per cigarette in commercial cigarettes.

The mainstream smoke of three brands of hand-rolled cigarettes from Thailand delivered 1.1–5.5 mg nicotine per cigarette (Mitacek et al. 1991).

It should be noted that there are no available data on nicotine in mainstream smoke of RYO delivered by more intense machine-smoking.

4.2 Cigars

There are many types of cigars on the market. In North America and in many parts of Europe, there are at least four types of cigars: little cigars, small cigars (also called cigarillos), regular cigars, and premium cigars. In 1997 in the USA, the leading

brands of little, large, and premium cigars (ranging in length from 7.3 to 17.6 cm and in weight from 1.24 to 8.1 g) were analyzed, and the levels of nicotine and selected carcinogens (e.g., BaP, NNN, and NNK) measured in the mainstream smoke (Djordjevic et al. 1997). The nicotine yields in the mainstream smoke of little, large, and premium cigars, as measured by the standard International Committee for Cigar Smoke Study method (puffs of 20 cm^3 taken every 40 s, duration of puffs 1.5 s; butt length 33 mm), were 1.5, 1.4, and 3.4 mg per unit, respectively. The levels of nicotine and NNK in the smoke of premium cigars were higher by three and 17 times, respectively, than in cigarette smoke of the best-selling cigarettes on the US market. When little filter-tipped cigars were machine smoked in a manner that mimicked human smoking behavior, the emissions of nicotine and TSNA were higher than those measured by the standard method. Thai cigars deliver 7.95–11.4 mg nicotine per unit in the mainstream smoke.

Seventeen brands of cigars ranging in weight from 0.53 to 21.5 g showed considerable variation in the total nicotine content of the tobacco: 5.9 to 335.2 mg per cigar (Henningfield et al. 1999). The aqueous pH of cigar tobacco ranged from 5.7 to 7.8. The smoke pH values of the smallest cigars were generally acidic, changed little across the puffs, and more closely resembled the profiles previously reported for tobacco of typical commercial cigarettes. The smoke pH of smaller cigars and cigarillos only became acidic after the first third of the rod had been smoked, and remained acidic thereafter. The smoke pH of larger cigars was acidic during the smoking of the first third of the rod, and became quite alkaline during the smoking of the last third. This phenomenon needs to be taken into consideration when evaluating the bioavailability and addictive potential of cigars.

In a study of 30 smokers of pipes or cigars only, 28 cigarette smokers only, and 30 nonsmoking male subjects matched for age, the urinary cotinine and 1-hydroxypyrene levels (a biological marker of exposure to carcinogenic polycyclic aromatic hydrocarbons; PAH) were found to be higher in cigarette smokers than in pipe or cigar smokers, and higher in the latter than in nonsmokers (Funk-Brentano et al. 2006). In multivariate analysis, cigarette smoking was the only independent predictor of CYP1A2 activity ($p < 0.0001$) and of 1-hydroxypyrene excretion in urine ($p = 0.0012$). In this study, pipe or cigar smoking was associated with lower exposure to products of tobacco metabolism than cigarette smoking, and to an absence of CYP 1A2 induction. However, inhalation behavior, rather than the type of tobacco smoked, may be the key factor linked to the extent of tobacco exposure and CYP 1A2 induction. It has been suggested that switching from smoking cigarettes to cigars, or smoking both products intermittently, may increase the exposure of smokers to toxic and carcinogenic agents (Henningfield et al. 1999). In contrast with “only cigar smokers” who relatively seldom inhale smoke into the lungs, former cigarette smokers and concurrent cigar and cigarette smokers have a tendency to maintain their cigarette smoke inhalation pattern when they smoke cigars, thus inhaling larger quantities of smoke toxicants.

4.3 Bidis (Hand-Rolled Indian Cigarettes) and Chutta (Hand-Made Indian Cigars)

The construction and appearance of bidi cigarettes differ markedly from commercial cigarettes. Bidis are manufactured primarily in India, and consist of about 150–250 mg of sun-dried tobacco (*N. tabacum*) flakes wrapped in a dried leaf of temburni (*Diospyros melanoxylon*) or tendi (*Diospyrosebenum*) (Pakhale and Maru 1998).

As reported by Malson and coauthors, the concentration of nicotine in the tobacco of 12 bidi cigarettes (mean value 21.2 mg g^{-1}; range 15.7–27 mg g^{-1}) was significantly greater than that in the tobacco from commercial filter-tipped cigarettes (16.3 mg g^{-1}) and unfiltered cigarettes (13.5 mg g^{-1}) (Malson et al. 2001). In ten smokers who switched to Irie bidi (strawberry-flavored cigarettes), plasma nicotine levels increased above the levels recorded when they smoked regular filter-tipped cigarettes (26 ng mL^{-1} versus 18.5 ng mL^{-1}) (Malson et al. 2002).

The amount of nicotine and nornicotine in Indian bidi tobacco was higher than that in Indian filter-tipped cigarettes (35.2 and 3.4 mg g^{-1}, respectively, versus 14.2 and 1.56 mg g^{-1}, respectively). Curiously, the mainstream smoke of Indian bidis delivered less nicotine than Indian cigarettes (1.87 mg per bidi versus 2.58 mg per cigarette) (Pakhale and Maru 1998).

A survey of the nicotine levels in the mainstream smoke from 21 brands of bidi cigarettes, both filtered and unfiltered, was conducted using a variation of the FTC standardized cigarette machine-smoking method (Watson et al. 2003). The primary difference between this method and the FTC method was a reduction of the 60-s puff interval to 15 s. The shorter puff interval was required to prevent the bidi cigarettes from self-extinguishing, and may represent a closer approximation to human usage. In this study, bidi cigarettes delivered 2.7 ± 0.4 mg nicotine per unit in the mainstream smoke. Unlike cigarettes, the filtered and unfiltered bidis delivered comparable smoke yields (2.82 ± 0.23 mg per bidi and 2.57 ± 0.22, respectively). When commercial cigarettes were machine-smoked using the same modified FTC method, nicotine deliveries ranged from 1.94 mg per cigarette (filtered brand) to 2.87 mg per cigarette (unfiltered brand).

A chutta is a type of a small hand-made cigar without a wrapper, and with a single tobacco leaf as a binder (Pakhale and Maru 1998). It consists of air-cured and fermented tobacco folded into a dried tobacco leaf. Chuttas usually do not have a filter, and are characterized by being open-ended. They are frequently associated with the remarkable habit of "reverse" smoking, during which the burning end is held inside the mouth (the reverse smoker inhales both the mainstream and the sidestream smoke). The nicotine content in chutta tobacco is comparable with that of bidi tobacco (30.84 mg g^{-1} versus 35.2 mg g^{-1}). However, the nicotine level in mainstream smoke from a chutta is higher (6.98 mg per product) than that from bidis (1.87 mg per product). The nicotine content in sidestream smoke of chutta was 2.07 mg per product.

4.4 Clove Cigarettes (kreteks)

Clove cigarettes are produced in Indonesia and exported throughout the world. They are composed of a mixture of tobacco (60–80%) and ground clove buds (20–40%), available with or without filters, and are usually machine-rolled in white, brown, or black paper. These cigarettes have a distinctive aroma because of the cloves. Eugenol, an analgesic, is naturally occurring in cloves, and is present in milligram quantities in the clove cigarette filler (Stanfill et al. 2006). Like menthol, eugenol diminishes the harshness of the tobacco smoke. The reported FTC/ISO nicotine yield in the mainstream smoke of clove cigarettes was 2 mg per unit (Malson et al. 2003).

4.5 Waterpipe Tobacco Smoking

The waterpipe (WP), also known as shiha, hookah, narghile, goza, and hubble bubble, has long been used for tobacco consumption in the Middle East, India, parts of Asia and, more recently, has been introduced into the smokeless tobacco market in western nations. WP smoking is so different from cigarette smoking that data on smoke composition and toxicity cannot be extrapolated from one to the other. Neergard and coauthors reviewed six studies to estimate daily nicotine exposure among adult WP users (Neergaard et al. 2007). These studies measured the nicotine or cotinine levels associated with WP smoking in four countries: Lebanon, Jordan, Kuwait, and India. Four of these studies directly measured nicotine or cotinine levels in human subjects. The remaining two studies used smoking machines to measure nicotine yield in smoke condensate generated by WP. In Lebanon, Shihadeh (2003) designed a first-generation smoking machine to determine the chemical profile of the WP mainstream smoke: 10 grams of tobacco smoked per session (100 puffs at 3 s per puff, 300 mL per puff, 30 s between puffs) generated 2.25 mg nicotine. Two years later, Shihadeh and Saleh (2005) reported a delivery of 2.94 mg nicotine per session under different machine-smoking conditions (171 puffs at 2.6 s per puff, 530 mL per puff, 2.8 puffs min^{-1}). The latter study reported high deliveries of CO (143 mg versus 1–22 mg per single cigarette) and the carcinogenic PAH (phenanthrene 0.748 μg versus 0.2–04; fluoranthene 0.221 μg versus 0.009–0.099; chrysene 0.112 versus 0.004–0.041). The source of high emissions of CO and PAH is the burning charcoal, which is normally placed atop the tobacco to smoke the narghile WP (Monzer et al. 2008).

Urinary cotinine values reported among WP users ranged from 0.184 μg mL^{-1} (at least three WP per week/14 males) to 6.08 μg mL^{-1} (at least one WP per day/15 males and one female) (Neergaard et al. 2007). To put it in perspective, cotinine values reported for users of other tobacco products were: cigarette smokers 1.28 μg mL^{-1} ($n = 12$; urine taken from an 8-h collection in people who smoked from 15–40 cigarettes per day); cigar smokers 0.67 μg mL^{-1} ($n = 8$); traditional pipe smokers 1.36 μg mL^{-1} ($n = 5$); and users of smokeless tobacco 0.92 μg mL^{-1} ($n = 9$) (Jacob III et al. 1999).

4.6 Potential Reduced-Exposure Products (PREPs)

Since the late 1980s, there has been a proliferation of new potential reduced-exposure products (PREPs), promoted by the industry with the claims of reduced harm, in all the four categories that were summarized earlier (Stratton et al. 2001; Hatsukami et al. 2002, 2005). These include (a) modified tobacco products, such as several denicotinized brands and reduced TSNA emission cigarettes; (b) chewing gum impregnated with tobacco; (c) smokeless tobacco products with claimed reduced nitrosamine levels; and (d) cigarette-like products (carbon-heated "smoking" devices).

Two prototypes of the new carbon-filtered Marlboro Ultra Smooth (MUS), marketed as a PREP, were investigated using both standard (FTC/ISO) and intensive (Health Canada) machine methods to measure gas/vapor and particulate phase smoke constituents (Rees et al. 2007). FTC nicotine yields in the mainstream smoke of two MUS varieties containing 180 and 120 mg carbon were 0.53 and 0.42 mg per cigarette, respectively, compared to the Marlboro Ultra Light brand, which delivered 0.56 mg nicotine per cigarette. Under more intense puffing conditions, nicotine emissions were 1.5, 1.32, and 1.6 mg per cigarette. The data suggest that MUS, although designed to reduce yields of select toxicants, preserved nicotine addiction and consumer appeal potential. Thus, claims made by the manufacturer for reduced harm status of MUS depend heavily on standard machine smoke yields, but not on clinical and long-term health outcome data. In June 2008, after 3 years of test marketing, Philip Morris pulled MUS off the market because it drew little attention from consumers.

Two versions of the reduced-nicotine cigarette Quest delivered 0.53 and 0.032 mg nicotine per cigarette under the standard FTC machine-smoking method (Chen et al. 2008). Historical research on low-nicotine cigarettes demonstrated that smokers compensated for lower nicotine delivery by increasing their puffing behavior to extract more nicotine (National Cancer Institute 2001). In a study by Strasser and coworkers, among 50 smokers of 0.6, 0.3, and 0.05 mg nicotine Quest cigarettes, total puff volume was greatest for the 0.05 mg nicotine cigarette and CO boost was moderately greater after smoking the 0.3 and 0.05 mg cigarettes compared to the 0.6 mg nicotine cigarette, suggesting that this product can potentially be a harm-increasing product (Strasser et al. 2007). Another study using research cigarettes with progressively reduced nicotine content (0.6–10.1 mg nicotine per gram tobacco; 0.1–0.8 mg in smoke per cigarette) showed that intake of nicotine declined progressively, with little evidence of compensation (Benowitz et al. 2007).

Smokers who switched to Advance, a cigarette with purportedly reduced levels of toxicants, were exposed to significantly higher levels of nicotine than when they smoked their own brand (23.3 ng mL^{-1} versus 18.6 ng mL^{-1}; $p < 0.05$) (Breland et al. 2002).

In 1997, R.J. Reynolds Tobacco Co. introduced Eclipse, a nicotine delivery device purported to deliver lower levels of smoke toxicants than conventional cigarettes. Eclipse uses a carbon fuel element to vaporize nicotine in the rod; the user then inhales the nicotine vapor. Venous plasma nicotine boost among ten smokers

was significantly lower 2 min after they smoked Eclipse than with their own brand (10.7 ng mL^{-1} versus 16.4 ng mL^{-1}) (Lee et al. 2004). Nevertheless, Eclipse exposes the user to significant quantities of nicotine, CO (7.3 versus 4.2 ppm from conventional cigarette), and possibly other harmful components of tobacco smoke.

5 Nicotine in Smokeless Tobacco Products

While cigarette sales in the USA declined 18%, from 21 billion packs in 2000 to 17.4 billion packs in 2007, during the same time period sales of other products, such as moist snuff, increased by 1.10 billion cigarette pack equivalents (Connolly and Alpert 2008). In the USA, the most common smokeless tobacco (ST) products are chewing tobacco (loose leaf, plug, and twist), moist snuff, and dry snuff. Many other forms of smokeless tobacco that are used globally were described in an IARC monograph (IARC 2007). All ST products contain nicotine and other tobacco alkaloids that are inherent to tobacco leaf.

In 1996, the Massachusetts Department of Public Health (MDPH) promulgated regulations that required cigarette and smokeless tobacco manufacturers to file annual reports on nicotine yield by brand (IARC 2004). Table 5 presents the mean values of total nicotine, pH, and unprotonated (free) nicotine for each type of ST product sold in the USA. On average, moist snuff contained the highest levels of total and free nicotine (2.58% dry weight and 3.52 mg g^{-1} of the product, respectively), followed by dry snuff (1.82% and 0.71 mg g^{-1}, respectively) and chewing tobacco (1.22% and 0.11 mg g^{-1}, respectively). The analysis of the MDPH database revealed the following: (a) free nicotine levels vary widely between brands, and are controlled by the manufacturer primarily by pH, consistent with well-known graduation strategies; (b) high market share products have high and/or increasing free nicotine; (c) there are increasing numbers of subbrands with designs to enhance or ease the delivery of nicotine; (d) a combination of the above factors, price discounts, clean indoor air policies, and other marketing strategies are most likely responsible for increasing moist snuff sales (Alpert 2008).

Table 5 Range of pH and nicotine concentrations in smokeless tobacco sold in Massachusetts (USA) in 2003 (IARC 2004)

Constituent	Chewing tobacco ($n = 74$) Mean (range)	Dry snuff ($n = 33$) Mean (range)	Moist snuff ($n = 106$) Mean (range)
Moisture (%)	22.8 (14.57–28.57)	8.2 (5.38–23.9)	52.6 (21.58–55.77)
Nicotine (%, dry wt)	1.22 (0.45–4.65)	1.82 (1.14–2.69)	2.58 (0.49–3.70)
Nicotine (mg g^{-1} product)	9.9 (3.41–39.74)	16.8 (10.48–24.84)	12.6 (4.7–24.29)
pH	5.82 (5.07–6.91)	6.36 (5.50–7.61)	7.43 (5.41–8.38)
Unprotonated (free) nicotine (mg g^{-1} product)	0.11 (0.02–1.77)	0.71 (0.05–3.12)	3.52 (0.03–8.57)

The free nicotine concept and its biological applications were discussed in detail in the peer-reviewed literature (Djordjevic et al. 1995; Hoffmann et al. 1995; Henningfield et al. 1995; Idris et al. 1991,1998; Fant et al. 1999; Ayo-Yusuf et al. 2004), as well as in an IARC monograph (IARC 2007) and in this book (see chapter: "Approaches, Challenges, and Experience in Assessing Free Nicotine," by Ashley et al., this volume). In countries such as South Africa, because of the high pH (up to 10.1) of popular commercial and traditional smokeless tobacco, the calculated percentage of free-base nicotine was reported at 99.1% (total nicotine up to 29.29 mg g^{-1} dry tobacco) (Ayo-Yusuf et al. 2004). In the Sudan, snuff, locally known as *toombak*, was introduced approximately 400 years ago (Idris et al. 1998). It is always processed into a loose, moist form, and its use is widespread in the country. Tobacco used for manufacture of *toombak* is of the species *N. rustica*, and the fermented ground powder is mixed with an aqueous solution of sodium bicarbonate. The resultant product is moist, has a strong aroma, is highly addictive, and its use is widespread, particularly among males. Its pH is 8–11, moisture content ranges from 6 to 60%, and nicotine content from 8 to 102 mg g^{-1} dry weight (Idris et al. 1991).

McNeill and coauthors analyzed the 11 smokeless products most popular in the UK, including Gutkha and Zarda varieties that have an origin in India, and four products purchased outside the UK (McNeill et al. 2006). The UK-purchased products contained very wide concentrations of nicotine (3.1–83.5 mg g^{-1}). Products purchased outside the UK included snus from Sweden (15.2 mg nicotine), Baba 120 from India (55 mg nicotine), and Copenhagen from the USA (9.2 mg nicotine). Some of the products purchased in the UK contained a very high pH (up to 9.94), resulting in very high concentrations of free nicotine (98.75%). Smokeless tobacco products available to consumers in India had pH ranging from 5.21 to 10.1, and contained 0.7–10.16 mg nicotine per gram of the product (Gupta 2004).

The latest analyses of new ST products marketed as PREPs on the US market, namely Philip Morris' Taboka and Marlboro Snus, R.J Reynolds' Camel Snus, and US Tobacco's Skoal Dry, revealed a very wide range of pH as well as total and free nicotine content: 6.47–7.75; 11.3–28.8 mg g^{-1} dry weight and 0.35–9.16 mg g^{-1}, respectively (Stepanov et al. 2008). These products are available in a variety of flavors, including "original," "green," "rich," "mild," "spice," "mint," "frost," "regular," "cinnamon," and "menthol." The highest pH and both total and free nicotine were measured in Camel Snus, and the lowest in Skoal Dry. The nornicotine content ranged from 0.31 to 1.04 mg. The comparison of contents of new PREPs and traditional forms of ST, such as Copenhagen, Skoal, and Kodiak (17.7–26.7 mg of total nicotine and 4.88–12.1 mg of free nicotine), show that PREPs have as high a nicotine content, and therefore have a propensity to initiate and sustain addiction among users.

Synthesized conclusions of the IARC monograph on smokeless tobacco (IARC 2007) and the opinion of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) on the health effects of smokeless tobacco SCENIHR (2008) can be summarized as follows: (a) all forms of ST are potentially addictive; (b) all forms of ST are carcinogenic; (c) there are probable reproductive health effects; (d) there are probable risk factors for myocardial infarction; (e) there is limited

and inconsistent evidence for ST as an effective smoking cessation aid; and (f) there is inconsistent association in patterns of smoking and ST use across countries.

6 Summary

The data presented in this chapter clearly show that all tobaccos used for factory- and hand-made products (e.g., Oriental, Virginia, burley, reconstituted tobacco sheets) all over the world contain nicotine and many other alkaloids. The concentrations of nicotine vary dramatically across different types of tobacco, and greatly depend on genetic potential, agricultural practices (including fertilization and plant density in the field), curing and processing methods, leaf position on the plant, storage practices, country of origin, and production year. The content of nicotine in tobacco products, both smoked and smokeless, largely depends on blending strategies, namely the types of tobaccos and their proportion in the blend, product design features, as well as additives that are used to enhance nicotine bioavailability and appeal to the user. It appears that all tobacco products contain enough nicotine to induce and sustain tobacco dependence. However, human exposure to nicotine does not depend solely on its content in the tobacco product and its design characteristics, but also on the way each individual uses the product, such as puffing intensity and filter vent-blocking among smokers, and the frequency and duration of dipping among smokeless tobacco users.

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Part II
Nicotine Pharmacology and Mechanisms of Action

The Road to Discovery of Neuronal Nicotinic Cholinergic Receptor Subtypes

Allan C. Collins, Outi Salminen, Michael J. Marks, Paul Whiteaker, and Sharon R. Grady

Contents

Abstract The discovery that mammalian brain expresses the mRNAs for nine different nicotinic cholinergic receptor subunits (α2–α7, β2–β4) that form functional receptors when expressed in *Xenopus laevis* oocytes suggests that many different types of nicotinic cholinergic receptors (nAChRs) might be expressed in the mammalian brain., Using an historical approach, this chapter reviews some of the

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progress made in identifying the nAChR subtypes that seem to play a vital role in modulating dopaminergic function. nAChR subtypes that are expressed in dopamine neurons, as well as neurons that interact with dopamine neurons (glutamatergic, GABAergic), serve as the focus of this review. Subjects that are highlighted include the discovery of a low affinity α4β2∗ nAChR, the identity of recently characterized α6* nAChRs, and the finding that these α6∗ receptors have the highest affinity for receptor activation of any of the native receptors that have been characterized to date. Topics that have been ignored in other recent reviews of this area, such as the discovery and potential importance of alternative transcripts, are presented along with a discussion of their potential importance.

1 Introduction

Binding sites in brain and autonomic ganglia for the nicotinic cholinergic receptor (nAChR) antagonist, α-bungarotoxin (α-Bgt), were first identified over 40 years ago (reviewed in Oswald and Freeman 1981). However, these binding sites were an enigma because virtually every study that had attempted to detect α-Bgt-induced blockade of cholinergic activities in brain preparations and autonomic ganglia during this time period had yielded negative results (reviewed in Schmidt 1988). Molecular biological approaches turned the nAChR field on its head with the discovery of 12 nAChR subunit genes that formed functional receptors when expressed in *Xenopus laevis* oocytes (reviewed in Lindstrom 1998). Those genes that coded for subunits that included two vicinal cysteines in the extracellular domain were designated alpha (α) subunits, while those that coded for subunits without the vicinal cysteines were termed non-alpha or structural subunits (terms that were ultimately replaced by the beta [β] designation). Mammalian brain expresses nine of these nAChR subunit genes (α2–α7, β2–β4) (Patrick et al. 1989; Heinemann et al. 1991; Lindstrom 1998). The remaining three subunits, α8–α10, are not expressed in mammalian brain (Schoepfer et al. 1990; Sgard et al. 2002; Keyser et al. 1993; Elgoyhen et al. 1994). The α and β subunits expressed in "peripheral-type" receptors (skeletal muscle and electric organs) are designated α1, β1, γ, δ and ε; α2–α10 and β2–β4 were assigned their names based on order of discovery.

This chapter summarizes progress made in identifying the subunit compositions of native nAChRs expressed in mammalian brain. The discovery that nine different subunits are expressed in the brain suggests that many, perhaps hundreds, of different nAChR subtypes might be expressed in the brain, assuming that neuronal nAChRs are made up of five subunits like the peripheral-type receptors (Karlin 2002). However, the number of subtypes that are actually expressed is certainly less than hundreds due to factors such as rules of receptor assembly and limitations on sites of expression. Nonetheless, the finding that *X. laevis* oocytes form functional receptors with varying biophysical and pharmacological properties when injected with cDNAs for the various subunits (Leutje and Patrick 1991; Chavez-Noriega et al. 1997) transformed the nAChR field dramatically. We went from zero functional receptors to perhaps dozens of potentially different nAChR subtypes.

Identifying the subunit compositions, sites of expression, and pharmacological properties of native receptors is of continuing interest and much of this progress has been summarized in two excellent recent reviews (Gotti et al. 2006a, 2007). We will provide an overview of this progress in this chapter, while paying particular attention to those nAChR subtypes that regulate the function of dopaminergic neurons. However, we will also highlight issues that did not receive much attention in the reviews by Gotti et al. For example, we will emphasize topics such as heteromeric α7-type nAChRs and the recently discovered low affinity α4β2* nAChRs (Marks et al. 2007) that are not discussed in the reviews by Gotti et al. We have opted to take a historical approach when describing this progress because history can often serve as a blueprint for future successes, and also because many of the individuals who studied the actions of nicotine and nAChRs have played important roles in the development of modern neuroscience. One of the rewards associated with writing this review was reading papers written by scientific giants such as Claude Bernard, John Langley, C.C. Chang, Michael Raftery, and Jean-Pierre Changeux and learning that discoveries made between 25 and over 100 years ago are still directly relevant to research being done today.

2 Receptive Substance, the Beginnings of a Field of Study

Virtually every ongoing study of nicotine recognizes that the actions of nicotine arise as a consequence of binding to and either activating or inhibiting (desensitization or channel block) the protein complex normally activated by acetylcholine (ACh). These receptors are also activated/inhibited by nicotine; hence the name nicotinic cholinergic receptors. The notion that nicotine interacts with a specific receptor dates back to a 1905 paper that is arguably the most famous nAChR paper ever published (Langley 1905). In this early paper, Langley reported that nicotine produces short-term stimulation followed by long-term blockade of both intact and denervated skeletal (striated) muscle and rightly concluded that nicotine interacts with a "receptive substance" expressed on or in skeletal muscles. He noted that the receptive substance is found at a site very near a "synaptic substance." Langley's receptive substance theory evolved into receptor theory, which is one of the basic tenets of modern biology. It should be noted that Langley advanced the receptive substance hypothesis 16 years before Otto Loewi (1921) demonstrated that the decrease in heart rate that follows electrical stimulation of the vagus is produced by release of a chemical from the vagus nerve (vagusstoff), 21 years before Loewi and Navratil (1926) demonstrated that vagusstoff is ACh, approximately 30 years before ACh was identified as the neurotransmitter at all sympathetic ganglia (Kibjakow 1933) and at the neuromuscular junction (Dale et al. 1936), and nearly 50 years before it was determined that ACh is the neurotransmitter at parasympathetic ganglia (Perry and Talesnik 1953).

Langley's receptive substance paper described studies done at the neuromuscular junction. In earlier studies he had demonstrated that nicotine affected the ganglia of

both the sympathetic and parasympathetic branches of the autonomic nervous system (Langley 1890; Langley and Dickinson 1889, 1890a), and that nicotine elicits short-term stimulation followed by longer-term blockade (paralysis) when applied to the autonomic ganglia (Langley 1901). It is also clear that Langley recognized that nicotine stimulates the central nervous system. For example, in his study that compared the actions of pituri and nicotine (Langley and Dickinson 1890b) Langley reported that "nicotine first stimulates and then paralyzes the central nervous system, and that it has in general a similar effect upon peripheral ganglia." Thus, by 1905, Langley had identified the three major sites of nicotine's actions and had postulated that all of nicotine's actions occur subsequent to interaction between nicotine and a receptive substance.

3 Pharmacological Approaches Identify Receptor Subtypes

Evolutionary biologists have argued for many years that selection pressures have favored the development of plants that produce poisons, such as nicotine, because these poisons decrease the likelihood that insects or animals will eat the plant. The recent finding that insects will eat tobacco (*Nicotiana attenuata*) genetically modified not to produce nicotine, certainly supports this popular assumption (Steppuhn et al. 2004). It is absolutely the case that if it were not for chemicals (drugs) that might be described as gifts from Mother Nature, the identification and characterization of the nAChRs would have been incredibly slow. We owe the initial discovery of most of these poisons to unknown ancient people (early pharmacologists) who learned to use poisons derived from plants and animals to "capture" food or to alter the inner being. It is scientifically correct to use the term "pioneering" in describing Claude Bernard's (1856) studies with curare and John Daly's (Badio and Daly 1994) more recent studies with epibatidine; however these eminent scientists did not discover the drugs that they used in their work. Unfortunately, the identities of the individuals who discovered these very important tools cannot be designated by: (Genius et al. 4002 BC).

3.1 Curare and Structure–Activity Analyses of Quaternary Ammonium Derivatives

Claude Bernard's early discovery (1856) that curare, the South American arrow poison (woorari), blocked muscle contraction elicited by stimulation of motor neurons, but not that elicited by direct stimulation of the muscle, provided the first demonstration that drugs could be used to study, what we now know are, nAChR-regulated functions. Bernard's early experiment served as the model for Langley's demonstration that the actions of nicotine on skeletal muscle could be blocked by pretreatment with curare (Langley 1880, 1907) and mimicked by pituri, the active

component of leaves from *Duboisia hopwood* that are chewed by Australian aborigines (Langley and Dickinson 1890b). These early studies established the concept of nicotinic agonists and antagonists and demonstrated that chemical structures might influence activity. Marshall (1913) established that the positively charged quaternary nitrogen found in most naturally-occurring nicotinic agents is vital for activity in his studies with tetraethyl ammonium (TEA). Nearly 40 years after Marshall's seminal findings, what might be viewed as follow-up structure–activity analyses of quaternary ammonium compounds resulted in the development of the bis-quaternary ammonium compounds $[(CH_3)_3N^+\text{-}(CH_2)_n - N^+(CH_3)_3]$ (Barlow and Ing 1948; Paton and Zaimis 1949). These first-ever structure–activity studies with nicotinic antagonists established that nAChR subtypes exist with the demonstration that ganglionic blockade is maximal when $n = 6$ (hexamethonium) and skeletal muscle blockade is maximal when $n = 10$ (decamethonium). Even today, the terms "C6" and "C10" are used to designate the ganglionic- and muscle-type nAChR subtypes.

3.2 α-Bungarotoxin (α-Bgt) and the Path to Identification of Neuronal Receptors

The need to develop better methods for treating snake bite, which had risen to epidemic proportions in Taiwan in the mid-twentieth century, led to the discovery that the "alpha" toxin derived from venom of the Taiwanese banded krait, *Bungarus multicinctus*, is a potent and irreversible inhibitor of electrical stimulation of the neuromuscular junction (Chang and Lee 1963). Subsequent studies showed that the toxin blocked carbamylcholine-induced depolarization of the electric organ of *Electrophorus electricus*; that these effects were blocked by pretreatment with *d*-tubocurarine, that the toxin blocked the binding of [^{3}H]-decamethonium to a protein extracted from the electric organ (Changeux et al. 1970); and that it had comparable effects at the neuromuscular junction (Miledi and Potter 1971). These results, coupled with the finding that α-Bgt did not block transmission in autonomic ganglia (reviewed in Schmidt 1988), added to the evolving data set that distinguished muscle-type receptors from ganglionic nAChRs.

α-Bgt provided an enormously powerful high-affinity tool that allowed purification and characterization of electroplaque (Heidmann and Changeux 1978) and skeletal muscle (Fambrough 1979) receptors. Purified receptors from these two sources were used to determine (among many things) that: (i) the nAChR is a pentameric assembly of four different subunits ($\alpha 1_2 \beta 1 \gamma \delta$ [neonatal] or $\alpha 1_2 \beta \varepsilon \delta$ [adult]); (ii) the α subunit contains a pair of disulfide-bonded cysteines that are separated by 13 amino acids (the Cys loop) in addition to a pair of vicinal cysteines that play a vital role in agonist binding; and (iii) each subunit has an N-terminal extracellular domain, four transmembrane domains, and two cyoplasmic loops between the first and second transmembrane domains (TM1–TM2), and a larger loop between TM3 and TM4 (reviewed in Karlin 2002). Purification of the four subunit proteins from *Torpedo californica* also allowed Raftery et al. (1980) to sequence the first 18 amino

acids of each of the four polypeptide chains obtained from *Torpedo* electric organ. Oligonucleotide probes based on the amino acid sequences were used to clone and then sequence the α1, β1 γ and δ subunit genes from the electric organs and skeletal muscle (Numa 1983). The peripheral-type nAChR gene sequences were then used to generate oligonucleotide probes that were used to clone and sequence the α2–α6 and β2–β4 subunits from rat brain (reviewed in Patrick et al. 1989; Heinemann et al. 1991) and α2–α4 and β2 from chick brain (Ballivet et al. 1988). Oligonucleotide probes generated from the amino acid sequence derived from an α-Bgt-binding protein isolated from chick brain (Conti-Tronconi et al. 1985) were used to clone the α7 and α8 cDNAs from chick brain (Schoepfer et al. 1990), α7 (Seguela et al. 1993) from rat brain, and both α9 (Elgoyhen et al. 1994) and α10 (Elgoyhen et al. 2001) from rat cochlear hair cells. Thus, α-Bgt played vital roles in identifying, cloning, and sequencing all of the known nAChR subunit genes.

4 Identification of α7* nAChRs

The α7-containing nAChRs were discovered earlier than the other neuronal nAChRs and are of enormous interest because of their unique properties (e.g., high permeability to Ca^{++}) and sites of expression.

4.1 [^{125}I]-α-*Bgt Binding*

Binding sites for [^{125}I]-α-Bgt had been described in autonomic ganglia (Patrick and Stallcup 1977) and in both mouse (Marks and Collins 1982) and rat (Clarke et al. 1985) brain, well before the nAChR subunit genes had been cloned and sequenced. The first report that [^{3}H]-nicotine binds with high affinity to rat brain also included the demonstration that α-Bgt did not block [^{3}H]-nicotine binding (Romano and Goldstein 1980). This, coupled with the findings that the regional distributions of [^{125}I]-α-Bgt and [^{3}H]-nicotine binding differ considerably in both mouse (Marks and Collins 1982) and rat (Clarke et al. 1985) brain, led to the conclusion that rodent brain expresses more than one nAChR subtype. Moreover, early pharmacological studies of [^{3}H]-nicotine binding (Romano and Goldstein 1980; Marks and Collins 1982) suggested that the brain nAChR(s) that bind nicotine with high affinity differ from the nAChRs found in autonomic ganglia, thereby raising the number of suspected nAChR subtypes to three.

4.2 α7 *mRNA Expression Patterns, in Situ Hybridization*

Early investigations of virtually all neuronal nAChR subunit genes, included in situ hybridization studies that determined mRNA expression patterns in rat [see, for

examples: α2 (Wada et al. 1988); α3 (Goldman et al. 1986); α4 (Boulter et al. 1986); α7 (Seguela et al. 1993); β2 (Deneris et al. 1988); β3 (Deneris et al. 1989)], chicken (Ballivet et al. 1988), and mouse (Marks et al. 1992) brain. Some of the mRNAs are expressed in both species in only very few brain regions (α2, β4), others (α3, α5) are readily identified in a significant number of brain regions, and others (α4, α7, β2) are expressed in many brain regions. The α6 (LeNovere et al. 1996; Azam et al. 2002) and β3 (Deneris et al. 1989; LeNovere et al. 1996; Azam et al. 2002) subunit mRNAs are expressed in very high concentrations in dopaminergic pathways as well as in visual pathways. Only a few brain regions (e.g., medial habenula and interpedunclear nucleus) seem to express virtually all the subunit mRNAs. These analyses suggest that some nAChR subtypes, particularly those that include α4, α7 and β2 subunits, might be broadly expressed in the brain, whereas others (e.g., α2β4) do not exist in appreciable numbers, if at all, in rodent brain.

The autoradiographic analyses of [^{125}I]α-Bgt binding done by Clarke et al. (1985) provided a very clear anatomical picture of brain regions that express the [^{125}I]-α-Bgt binding sites. These binding data were compared with the in situ hybridization patterns of the subunit mRNAs in virtually all "subunit discovery" papers. These comparisons are confounded if protein products are expressed in nerve terminals, given that mRNA is expressed principally in cell bodies. Nonetheless, Chen and Patrick (1997) were correct when they concluded that the α-Bgt-binding nAChRs are made up of α7 subunits when they noted that regional expression patterns for α7 mRNA and [^{125}I]-α-Bgt binding are very similar in rat brain. This result, coupled with the finding that both chick (Couturier et al. 1990) and rat (Seguela et al. 1993) α7 cDNA injected into *Xenopus* oocytes produced functional, homomeric receptors that were blocked by α-Bgt, led to the conclusion that the α-Bgt-binding nAChR is made up solely of α7 subunits. The assertion that α7 subunits are absolutely required to form the α-Bgt-binding receptor is proved by the finding that [^{125}I]-α-Bgt binding is absent in brain from α7 null mutant (gene knockout) mice (Orr-Urtreger et al. 1997). This conclusion is supported by findings that polyclonal antibodies directed against the α7 subunit detect only α7 subunits in rat brain (Chen and Patrick 1997) and affinity purification of PC12 cell-derived nAChRs yields only one type of subunit, although two-dimensional electrophoreses uncovered seven 35 kDa spots that differed in charge (pI value), which might reflect differences in posttranslational processing (Drisdel and Green 2000). The effects of these posttranslational modifications, if any, on receptor properties have not been determined.

4.3 Heteromeric α7 Receptors*

The first paper that described the cloning and sequencing of the α7 nAChR subunit gene from chicken brain identified two cDNA clones encoding two α-bungarotoxin–binding proteins, designated αBgtBP1 and αBgtBP2 (Schoepfer et al. 1990). Consistent with sequencing data, subunit-specific antibodies precipitated two receptor subtypes: approximately 85% included only αBgtBP1, the

remaining 15% contained both αBgtBP1 and αBgtBP2. Keyser et al. (1993) subsequently demonstrated that αBgtBP1 is the α7-encoded protein and αBgtBp2 the α8-encoded protein. Thus, approximately 15% of total α7* nAChRs formed in chick brain are α7α8 heteromers with unknown stoichiometry. When expressed in *Xenopus* oocytes, nAChRs that include the α8 subunit have lower affinity for α-Bgt (faster dissociation) and higher affinity for most agonists, including ACh and nicotine, than do homomeric α7 receptors (Anand et al. 1993).

Two groups (Yu and Role 1998a, b; Sudweeks and Yakel 2000) have speculated that heteromeric α7* nAChRs might exist, based on the findings that receptor function measured using native tissues differs dramatically from homomeric α7 receptors expressed in *Xenopus* oocytes. Yu and Role (1998a,b) suggested that chick autonomic ganglia may express α7α5 heteromeric nAChRs because the functional properties of chick ganglionic receptors that they measured differed from the functional properties reported for α7 homomeric receptors expressed in *Xenopus* oocytes and because these differences were lost following treatment with α5 antisense oligounucleotides. Given that immunoprecipitation techniques using subunit-specific antibodies do not detect α7α5 heteteromers (Pugh et al. 1995; Cuevas and Berg 1998), it does not seem likely that α7α5 receptors exist, at least in large numbers. It is likely (see Sect. 4.2) that heteromeric nAChRs made up of α7–1 and α7–2 alternative transcripts explain the data that prompted Yu and Role (1988a ,b) to suggest that α7α5 nAChRs might be formed in autonomic ganglia.

Yakel and colleagues (Sudweeks and Yakel 2000; Khiroug et al. 2002) also used functional data as the basis for their speculation that rat brain might produce α7β2* nAChRs. This argument was based on the findings that most rat brain regions that express the mRNA for α7 also express β2 mRNA (Sudweeks and Yakel 2000) and because α7 and β2 subunits coassemble to form receptors in oocytes with functional properties that resemble those of the α7* nAChRs expressed in hippocampal neurons (Khiroug et al. 2002). However, β2 gene deletion does not alter mouse brain [^{125}I]-α-Bgt binding (Zoli et al. 1998; Whiteaker et al. 2000) or a component of [^{3}H]-epibatidine binding that requires the α7 subunit (Marks et al. 2006). Further, no studies that have used antibodies directed against the β2 subunit have shown that α7 is precipitated along with the β2 subunit. Thus, the bulk of published data does not support the suggestion that heteromeric receptors made up of α7 and one or more of the other known α or β subunits are actually produced in autonomic ganglia or brain of mammals.

4.4 Alternative Transcripts and α7 Receptors*

The literature rarely, if ever, includes discussions that suggest that native heteromeric α7-type receptors might exist, even though early attempts at purification using α-Bgt (e.g., Conti-Tronconi et al. 1985) detected four α-Bgt-binding proteins with molecular weights ranging between 48,000 and 72,000. Reluctance to pursue the notion of heteromeric α7-type receptors may reflect the fact that several studies have presented compelling evidence that supported the conclusion that all α7-type

receptors are made up of α7 subunits only (Chen and Patrick 1997; Drisdel and Green 2000). We are persuaded that different types of α7* receptors might exist, at least in mouse brain, based on the results of a series of studies that have evaluated the effects of chronic nicotine or chronic glucocorticoid treatment on brain [^{125}I]-α-Bgt binding. We have reproducibly found that regulation of α7* receptor expression varies dramatically across brain regions following chronic nicotine treatment (see Marks et al. 1983, Pauly et al. 1991 for examples). Nicotine-induced increases (up-regulation) in mouse brain [^{125}I]-α-Bgt binding are dose-dependent, occur at higher doses than are required to produce an increase in [^{3}H]-nicotine binding, and vary dramatically across brain regions, with the hippocampus showing unique sensitivity. Similarly, mouse brain regions vary dramatically (hippocampus is the most sensitive) in chronic corticosterone-induced decreases in [^{125}I]-α-Bgt binding (Pauly et al. 1990a; Pauly and Collins 1993) and adrenalectomy-induced increases in α-Bgt binding (Pauly et al. 1990b). A convenient, but totally untested, explanation for these findings is that not all brain regions express identical α7* nAChRs.

Alternative transcripts provide one potential explanation for the apparent heterogeneity in regulation of α7 expression across brain regions. Severance et al. (2004) have recently shown that alternative transcripts for α7 (designated α7–1 and α7–2) are expressed in rat autonomic ganglia and brain, and that receptors formed from these alternative transcripts have functional properties that resemble those seen with α7α8 heteromeric receptors. The α7–2 isoform includes an 87 base-pair cassette that is inserted in the exon that codes for the N-terminus of the α7–1 isoform (the "standard" isoform). When expressed in *Xenopus* oocytes, the α7–2 isoform produced receptors that desensitize slowly and exhibit a readily-reversible α-Bgt blockade. These properties closely resemble the properties of α7α8 nAChRs expressed in oocytes (Anand et al. 1993) and chick ganglionic α7* receptors (Yu and Role 1998a). The protein products for both α7–1 and α7–2 are expressed in all the brain regions that express α7 mRNA. Thus, mammalian brain may produce, using alternative transcripts, α7* receptors that serve the same purpose as α7α8 nAChRs do in chick.

Mouse brain also expresses at least two alternative transcripts for α7 (Saragoza et al. 2003). The nontraditional mouse α7 transcript, like α7–2 from rat, produces changes in the N-terminal domain. In this case, if produced, the variant protein would have a single amino acid substitution in the N-terminal domain. However, the unique transcript also contains an extra exon that arises from alternative splicing of intron 9. The protein product resulting from this alternatively processed RNA is truncated shortly after the third transmembrane domain. The alternatively spliced protein product acts as a dominant negative (i.e., inhibitor) of the α7 function when expressed along with the standard α7 in GH4C1 cells. Though highly speculative, it may be that heterogeneity across mouse brain regions in expression of receptors that include proteins derived from alternative transcripts, might explain why chronic drug-induced changes in [^{125}I]-α-Bgt binding differ so dramatically across mouse brain regions.

4.5 Sites of Expression and Function of α7 Receptors*

[^{125}I]-α-Bgt binding is found in many regions of both rat (Clarke et al. 1985) and mouse (Pauly et al. 1989) brain. Binding is particularly high in the hippocampus, where it is found on what are likely to be GABAergic interneurons in the stratum oriens and stratum radiatum, and on pyramidal neurons. Many of the α7* receptors are expressed somatodendritically on some, but not all, GABAergic interneurons (see, for examples Alkondon et al. 1999; Frazier et al. 1998; Zhang and Berg 2007). α7* nAChRs are also expressed on dendrites and cell bodies of some dopaminergic neurons in the ventral tegmental area (Wu et al. 2004). Functional and immunocytochemical data indicate that α7* nAChRs are expressed on the terminals of some, but not all, neurons that use glutamate as a neurotransmitter in hippocampus and VTA (Gray et al. 1996; Mansvelder and McGehee 2000; Fabian-Fine et al. 2001; Jones and Wonnacott 2004). These findings have sparked research that is geared towards understanding the role of α7* nAChRs in modulating learning and memory and addiction processes, and in the development of drugs that might be used to treat pathologies that are due to altered function of pathways that express these receptors.

5 Heteromeric Receptors Containing α4 and β2 Subunits: α4β2*

The earliest studies that attempted to determine whether α4 subunits formed functional receptors in *Xenopus* oocytes established that function was obtained only if the oocytes were also injected with either β2 or β4 cDNA, thereby establishing the concept of heteromeric neuronal nAChRs (Deneris et al. 1988, Connolly et al. 1992). The α4β2* nAChR has been studied extensively because: (i) it seems to be the most widely expressed nAChR subtype; (ii) it was considered, until recently, to be the highest affinity nAChR; and (iii) the number and function of these receptors are altered by chronic nicotine treatment.

5.1 Ligand Binding, In Situ Hybridization, and High Affinity α4β2 Receptors*

Early comparisons of α4 and β2 mRNA expression patterns and [^{3}H]-nicotine binding (Boulter et al. 1986; Deneris et al. 1988; Marks et al. 1992) suggested that nAChRs including these two subunits make up what was termed “high affinity” nicotine binding sites. This conclusion was accepted with suspicion however, because several brain regions have mismatches between binding and mRNA expression. Eventually much of this mismatch could be attributed to the fact that α4 and β2 mRNA are expressed in cell bodies, whereas many α4β2* nAChRs are expressed on nerve terminals where they modulate neurotransmitter (GABA and dopamine)

release. Other inexplicable mismatches between binding and mRNA expression still abound. The most notable of these exceptions is cerebellum, where massive levels of β2 mRNA are found with little or no detectable binding in mouse brain. This expression is not an artifact given that no cerebellar signal is detected in β2 null mutants (Picciotto et al. 1995). To this day, no one has provided a reasonable explanation for the massive expression of β2 mRNA in cerebellum, nor has a suitable α subunit that might be coexpressed with the β2 subunit been identified. As a result, the notion that α4 and β2 subunits make up the high affinity nicotine binding site was not generally accepted until it was shown that null mutation of both the α4 (Marubio et al. 1999) and β2 (Picciotto et al. 1995) subunit genes resulted in elimination of the [^{3}H]-nicotine binding site.

The [^{3}H]-nicotine binding sites, and α4β2* nAChRs, have been referred to for nearly 30 years as the high affinity nicotine receptor, a term used by Romano and Goldstein (1980) because the dissociation constant (K_d) for nicotine binding to rat brain membranes is in the low nanomolar range. Similar results are obtained in mouse brain; e.g., we consistently calculate K_d values of 2–5 nM for nicotine to mouse brain membranes (Bhat et al. 1994; Marks et al. 1986, 1991, 1992). The high affinity nicotine binding site is also referred to as the high affinity agonist binding site because other nicotinic agonists such as [^{3}H]-cytisine (Pabreza et al. 1991) and [^{3}H]-ACh (Schwartz et al. 1982) bind to the same sites as does nicotine in mouse (Marks et al. 1986) and rat (Pabreza et al. 1991) brain. Romano and Goldstein (1980) argued that nicotine binds to the high affinity, desensitized form of the receptor because the K_d values obtained in their studies were ten- to 100-fold less than EC_{50} values for receptor activation. Kinetic analyses of [^{3}H]-nicotine binding demonstrated that association rates are biphasic, which provided support for this postulate (Lippiello et al. 1987; Bhat et al. 1994). However, the best support for this postulate comes from the finding that the EC_{50} values for nicotine stimulation of current flow by α4β2 nAChRs expressed in *Xenopus* oocytes are approximately 100-fold higher than the K_d values determined for binding (Leutje and Patrick 1991; Connolly et al. 1992; Sabey et al. 1999; Rush et al. 2002) and the finding that subactivating concentrations of nicotine can fully desensitize α4β2 nAChRs with an EC_{50} value that is very similar to the K_d values reported for binding (Fenster et al. 1997). However, recent studies have shown that receptors that include only the α4 and β2 subunits are not the highest affinity nAChRs when receptor activation is measured. nAChRs that include α6 and β3 subunits, along with α4 and β2, have the lowest EC_{50} values of any native nAChRs that have been measured to date (Salminen et al. 2007).

5.2 [^{3}H]-Epibatidine Identifies Low Affinity α4β2 Receptors*

A little over 10 years ago radiolabeled epibatidine (Houghtling et al. 1995) was introduced as a new ligand with extraordinarily high affinity for α4β2* nAChRs. Early reports suggested that [^{3}H]-epibatidine binds to α4β2-type receptors only, but this assertion was quickly questioned when it was noted that epibatidine binding exceeds

that of [^{3}H]-agonist binding in several brain regions, and is present in some brain regions (optic nerve, optic chiasm, optic tract) that have no detectable [^{3}H]-agonist binding sites (Perry and Kellar 1995). This concern was enhanced by the observation that β2 gene deletion does not eliminate [^{3}H]-epibatidine binding in several brain regions that do not bind nicotine with high affinity (Whiteaker et al. 2000). As shown in Fig. 1a, saturation studies that use a broader range of ligand concentrations than were used in early [^{3}H]-epibatidine binding studies yield data indicating that more than one binding site exists. The biphasic nature of [^{3}H]-epibatidine binding can be readily seen when binding data are plotted using the Scatchard transformation (insert to Fig. 1a); the data yield the "hockey stick" shape that is characteristic of two sites that differ in affinity for the ligand. Epibatidine binding can be separated into higher (K_d = 10–20 pM) and lower (K_d = 10 nM) affinity classes; the ratio of these two major classes varies dramatically across brain regions (Marks et al. 2000).

Figure 1 also shows that the high and low affinity [^{3}H]-epibatidine binding sites can be further subdivided on the basis of sensitivity to inhibition by other nicotinic compounds. For example, Fig. 1b shows that cytisine is a potent inhibitor of [^{3}H]-epibatidine binding (results using 0.3 and 10 nM are shown). Note that more than 4 log units of cytisine concentration is required to attain total inhibition, a result that predicts that more than one binding site is being measured in these assays. Indeed, a two-site model provides the best fit to the inhibition data. This prompted us to use the terms cytisine-sensitive and cytisine-resistant to describe these two components of higher affinity [^{3}H]-epibatidine binding. Null mutation of both the α4 and β2 genes results in near-total elimination of the cytisine-sensitive component of higher affinity epibatidine binding, thereby demonstrating that these binding sites measure α4β2* nAChRs (Marks et al. 2006, 2007). Deletion of the α7, β4 (Marks et al. 2006), and α5 (Brown et al. 2007) subunits does not produce a detectable change in cytisine-sensitive higher affinity [^{3}H]-epibatidine binding. In contrast, a substantial fraction of the cytisine-resistant component is eliminated by β4 gene deletion (i.e., cytisine-resistant higher affinity [^{3}H]-epibatidine binding can be used to measure β4-containing nAChRs).

Figure 1c shows that the lower affinity site can be separated into components that are more or less sensitive to inhibition by *d*-tubocurarine (Marks et al. 1998; Whiteaker et al. 2000). Studies that evaluated the effects of gene deletion (i.e., null mutant analyses) on lower affinity binding yielded some results that were fully expected. For example, approximately 30% of the lower affinity binding sites are eliminated by α7 gene deletion and are blocked by α-Bgt (Marks et al. 2007). To our surprise, most (approximately 75%) of the remaining lower affinity [^{3}H]-epibatidine binding sites are eliminated throughout the brain by β2 (Marks et al. 2006) and α4 (Marks et al. 2007) gene deletion, indicating that these are α4β2* nAChRs that have low affinity for agonists. These low affinity [^{3}H]-epibatidine binding sites are found throughout the brain in numbers that are nearly equal to the high affinity [^{3}H]-nicotine binding sites that were first identified in the early 1980s (Romano and Goldstein 1980, Marks and Collins 1982; Clarke et al. 1985). The reports that α4 (Marubio et al. 1999) and β2 (Picciotto et al. 1995) eliminated the high affinity

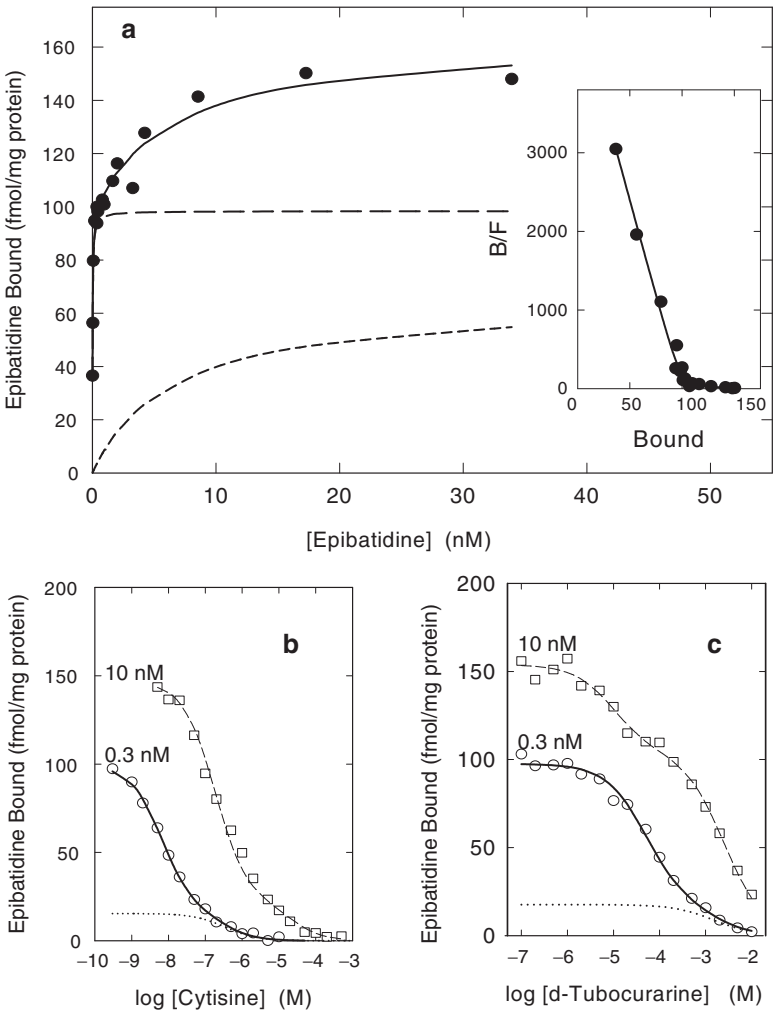

Fig. 1 Binding of [^{3}H]-epibatidine to membranes prepared from mouse brain. **a** depicts results of an experiment where varying concentrations of [^{3}H]-epibatidine were incubated with membranes prepared from whole mouse brain under equilibrium binding conditions (see Marks et al. 2006, 2007 for specifics of the assay). As concentration increased, saturation was achieved, but as is most readily seen by Scatchard analysis (*inset*); the data were best fit by a two-site model. **b** (higher affinity) and **c** (lower affinity) provide the results of competition binding experiments. The data presented in **b** show that the addition of varying concentrations of unlabeled cytisine to incubations that contained either 0.3 nM [^{3}H]-epibatidine (a concentration that fully saturates the high affinity epibatidine binding site) or 10 nM [^{3}H]-epibatidine (saturates the low affinity site) results in total inhibition of binding. However, more than 4 log units of cytisine were required to completely inhibit binding, leading to the conclusion that [^{3}H]-epibatidine binds to at least two nAChR subtypes that differ in affinity for cytisine (i.e., cytisine-sensitive and cytisine-resistant). **c** depicts the results of similar experiments that used *d*-tubocurarine to inhibit [^{3}H]-epibatidine binding

nicotine binding site had led to the apparently erroneous conclusion that all α4β2* nAChRs are always high affinity nAChRs. This clearly is not the case. At this point, all that is unequivocally known about these low affinity sites is they require both α4 and β2 and are found throughout the brain.

It is also the case that the function of these binding sites is unknown, although it may be that low affinity epibatidine binding is measuring low affinity receptors that have been detected in mouse brain synaptosomal preparations using ion ($^{86}Rb^{+}$) flux assays (Marks et al. 1999). Figure 2 shows that agonist-induced $^{86}Rb^{+}$ flux can be separated into high and low affinity components (4 log concentrations are required to elicit maximal ion flux and the data are best fit by a two-site model) (Marks et al. 1999, 2000, 2007). It is clear (Fig. 2) that both components are modulated by α4β2* nAChRs given that both α4 and β2 gene deletion eliminate both the high and low affinity components (Marks et al. 1999, 2007). An analysis of both components of binding and ion flux that included 12 brain regions found a significant correlation between high affinity binding and high affinity agonist-induced ion flux and a

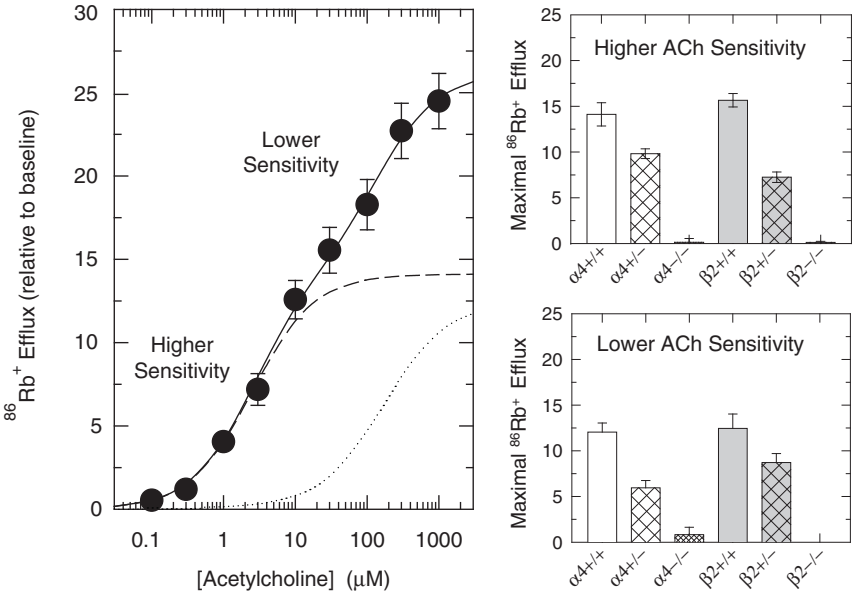

Fig. 2 Agonist-stimulated $^{86}Rb^{+}$ from mouse brain synaptosomes. Acetylcholine (ACh)-stimulated ion ($^{86}Rb^{+}$) efflux from synaptosomes prepared from mouse brain was done as described in Marks et al. (2007). The *left panel* of this figure demonstrates that 4 log units of agonist (ACh) were required to elicit maximal ion flux. These data are fit best by a two-site model indicating that higher and lower sensitivity components of the ion flux response exist. The *right hand panels* of the figure illustrate the effects of α4 and β gene deletion on the ion flux responses. Both α4 and β2 gene deletion resulted in total elimination of both the higher and lower sensitivity components of the ion flux response to ACh. ACh-stimulated release from synaptosomes prepared from mice that were heterozygous for the null mutations ($\alpha4^{+/-}$ and $\beta2^{+/-}$) showed intermediate levels of ion flux. These results demonstrate that α4β2* nAChRs are responsible for both components of the ion flux response

similar significant correlation between low affinity binding and low affinity ion flux (Marks et al. 2007). This finding suggests that the low affinity binding site may be measuring the same nAChR subtype(s) that modulates the low affinity component of ion flux.

Biphasic dose–response curves for agonist-induced increases in current flow and epibatidine binding have also been described for α4β2 nAChRs expressed in cell lines and *Xenopus* oocytes (Zwart and Vijverberg 1998; Buisson and Bertrand 2001; Nelson et al. 1992; Zhou et al. 2003). The expression system studies attempted to manipulate α4 and β2 subunit levels by altering mRNA ratios and by using receptors with α4 and β2 subunits linked together (concatamers). The results indicate that the high affinity components of binding and flux may be measuring nAChRs with two copies of α4 and three of β2 ($\alpha4_2\beta2_3$), and that lower affinity binding and flux may be measuring receptors made up of three α4 and two β2 subunit ($\alpha4_3\beta2_2$). Recently, we (Gotti et al. 2008) have reported results of experiments done with heterozygous α4 and β2 null mutant mice (i.e., $\alpha4^{+/-}$ and $\beta2^{+/-}$) that support the suggestion that $\alpha4_2\beta2_3$ and $\alpha4_3\beta2_2$ nAChRs are both found in mouse brain. Specifically, the ratios of the high and low affinity components of ACh-stimulated $^{86}Rb^{+}$ efflux as well as α4 and β2 protein expression are affected by the altered ratios of both α4 and β2 mRNAs that are seen in $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice.

5.3 Heteromeric α4β2 Receptors that Include Other nAChR Subunits*

At least two heteromeric α4β2* nAChRs that include additional nAChR subunits have been identified to date. It is readily apparent from in situ hybridization studies that not all α4β2 nAChRs could possibly include the α5 subunit because α5 mRNA expression is limited when compared with α4 and β2 mRNA expression. However, RT-PCR studies have detected coexpression of the α4, α5, and β2 mRNAs in GABAergic neurons from rat cortex (Porter et al. 1999) and striatum (Klink et al. 2001). Recently, we (Brown et al. 2007) reported that subunit-specific antibodies will precipitate α4α5β2 nAChRs in approximately half of the 12 mouse brain regions that we studied. Ligand binding, antibody, and functional data have demonstrated that α4α5β2 nAChRs are expressed in dopamine neurons (see Sect. 6) and other (unpublished) work from our laboratory indicates that GABA release is modulated by α4α5β2 nAChRs in some, but not all, mouse brain regions. In addition, it has been established that some α4β2* nAChRs in dopaminergic neurons contain α6 and β3 subunits (see Sect. 6).

5.4 Alternative α4 Transcripts

One of the first studies that described the functional properties of α4β2 nAChRs indicated that rat brain forms two α4 transcripts, designated α4–1 and α4–2 (Connolly

et al. 1992). The C-terminal end of the α4 gene differs in these two alternate splice variants. The only report that describes the potential importance of these two transcripts suggests that receptors that include the C-terminal sequence coded for by the α4–2 transcript (same C-terminal as the human transcript) have increased sensitivity to the allosteric actions of some steroids (Paradiso et al. 2001).

5.5 Polymorphisms in the α4 and β2 Genes

Naturally occurring single nucleotide polymorphisms have been identified for virtually all of the human neuronal nAChR subunits, but most of these have not been studied in detail. Notable exceptions are the α4 and β2 polymorphisms that are linked with a rare form of epilepsy, autosomal dominant nocturnal frontal lobe epilepsy, ADNFLE (Steinlein et al. 1995; Phillips et al. 2001; De Fusco et al. 2000; Combi et al. 2004; Hogg and Bertrand 2004). Several linkage analyses have detected significant genetic associations between ADNFLE and α4 or β2 polymorphisms (Weiland et al. 2000; Steinlein 2007). These associations are provocative, especially since the mutant α4 or β2 genes have altered receptor function when expressed in vitro along with native β2 or α4 genes, respectively (Kuryatov et al. 1997; De Fusco et al. 2000; Rodriguez-Pinguet et al. 2005; Bertrand et al. 2005). More recently, mice with some of these polymorphisms have been generated and the results indicate that such mutations may be sufficient to cause phenotypic changes similar to ADNFLE (Klassen et al. 2006; Xu et al. 2006; Teper et al. 2007).

6 Receptor Subtypes Expressed in Dopamine Neurons

Identifying and characterizing the nAChR subtypes expressed in dopaminergic neurons has been of primary interest, principally because dopaminergic systems presumably play a vital role in modulating the reinforcing effects of nicotine. Rapid progress has been made in this area in recent years and a very complex story has emerged: a minimum of five different nAChR subtypes are expressed in dopamine neurons.

6.1 [^{3}H]-Epibatidine Binding and mRNA Expression

Techniques that measure mRNA expression and ligand binding assays that measure receptor expression have been used extensively to identify those nAChR subtypes that are expressed in dopamine neurons. In situ hybridization studies using mouse (Marks et al. 1992; Grady et al. 1997) and rat (Le Novere et al. 1996) brain have detected the mRNAs for all of the known nAChR subunits, except α2 and β4, in

brain regions that are rich in dopamine cell bodies, such as the substantia nigra and ventral tegmental area. Techniques designed to measure mRNA in specific cell types have been used in other studies to identify those mRNAs that are expressed in dopamine neurons, in part because dopamine-rich brain regions also contain many GABAergic neurons that also express nAChRs. These methods, double-label in situ hybridization (Azam et al. 2002) and single-cell RT-PCR (Klink et al. 2001), have detected α4 and β2 mRNAs in virtually every dopaminergic cell body. A very high fraction (70–80%) also express α5, α6, and β3 mRNAs and approximately half of the dopamine neurons express α3 and α7 mRNAs. These findings suggest that dopamine neurons may express many different nAChR subtypes.

Ligand binding assays done with brain tissue obtained from nAChR subunit null mutant mice have provided critical data that have helped identify the subunit compositions of those nAChRs that are actually expressed in dopamine-rich brain regions and in dopaminergic neurons. Membrane-binding studies done with [^{3}H]-epibatidine as the ligand and brain tissue derived from α4, α5, α7, β2, and β4 null mutant mice have demonstrated that high levels of both high and low affinity α4β2* and intermediate levels of α7* nAChRs are expressed in dopamine-rich regions of mouse brain (Marks et al. 2006; 2007). These assays, while informative, measure all of the receptors that are expressed in these brain regions.

6.2 Binding and Functional Studies Using α-Conotoxin MII

Binding studies with radiolabeled α-conotoxin MII (α-CtxMII) have yielded the most informative results to date. These studies were built on the discovery that α–CtxMII binds with high affinity to, and blocks the activation of, α3β2* nAChRs (Cartier et al. 1996) and α6β2* nAChRs (Kuryatov et al. 2000) expressed in *X. laevis* oocytes. These observations, coupled with the demonstration that α-CtxMII blocks the release of [^{3}H]-dopamine from both rat (Kulak et al. 1997) and mouse (Grady et al. 2002) striatal synaptosomes suggest that α3β2* nAChRs (Cartier et al. 1996) and α6β2* nAChRs might be expressed in dopamine nerve terminals. Given that treatment with the dopamine neuron neurotoxin, MPTP, results in decreases in mouse striatal [^{125}I]-α-CtxMII binding that closely parallel declines in dopaminergic but not GABAergic markers, it seems highly likely that α-CtxMII-binding nAChRs are expressed almost exclusively in dopaminergic neurons (Quik et al. 2003).

Studies that evaluated the effects of nAChR gene deletion on [^{125}I]-α-CtxMII binding have helped identify the subunit compositions of α–CtxMII-binding receptors that are actually expressed in brain. Autoradiographic analyses showed that null mutation of the α6 (Champtiaux et al. 2002) subunit gene results in total elimination of [^{125}I]-α-CtxMII binding in dopaminergic pathways (Champtiaux et al. 2002), but α3 gene deletion has no effect (Whiteaker et al. 2002). The lack of effect of α3 null mutation is somewhat surprising given that the mRNA for this subunit is expressed in many dopaminergic neurons (Klink et al. 2001; Azam et al. 2002).

The finding that α3 gene deletion eliminates [^{125}I]-α-CtxMII binding in some brain regions (e.g., medial habenula, fasciculus retroflexus) and reduces binding in others (e.g., interpedunclear nucleus) (Whiteaker et al. 2002) demonstrates that [^{125}I]-α-CtxMII binds to native α3-containing nAChRs with high affinity. Thus, it may be that α3-containing nAChRs are not formed in dopaminergic neurons or it may be that α3* nAChRs are normally formed in dopaminergic neurons, but are replaced by α6*-AChRs in α3 null mutant mice.

The effects of α4, α5, α7, β2, β3, and β4 null mutation on [^{125}I]-α-CtxMII binding have been measured using membranes prepared from regionally dissected mouse brain (Salminen et al. 2005). Deletion of α5, α7, and β4 do not alter the binding of [^{125}I]-α-CtxMII to striatal membranes. In contrast, null mutation of β2 causes near-total loss of [^{125}I]-α-CtxMII binding in striatal membranes, indicating that most, if not all, of the nAChRs that bind [^{125}I]-α-CtxMII with high affinity require both the α6 and β2 subunits for their formation. Deletion of the α4 subunit results in a 50–75% decrease in [^{125}I]-α-CtxMII binding from striatal membranes, indicating that some α6β2* nAChRs include the α4 subunit (i.e., α4α6β2*). Deleting the β3 gene also results in a marked (approximately 65%) decrease in [^{125}I]-α-CtxMII binding, indicating that α4α6β2β3, α6β2β3, and α6β2 nAChRs are expressed in dopaminergic neurons in the mouse (see Fig. 3).

6.3 Immunological Approaches

Immunological approaches have been used to verify which subunits combine to form a receptor subtype. Champtiaux et al. (2003) used antibodies directed against rat and human α4–α7 and β2–β4 subunits in immunoprecipitation experiments to identify three heteromeric receptors α4β2*, α4α6β2*, and α6β2* in striatum. Gotti et al. (2005) identified α4α6β2β3, α6β2β3, and α6β2 subtypes in a study that evaluated the effects of β3 null mutation on [^{3}H]-epibatidine binding that was precipitated by these same antibodies. Similar immunological methods have identified all of these α6-containing receptors in striatal tissue obtained from rat (Zoli et al. 2002), squirrel monkeys (Quik et al. 2005), and humans (Gotti et al. 2006). A very recent report that used subunit-specific antibodies and α5 null mutant mice to demonstrate that many α4β2* nAChRs also contain the α5 subunit (Brown et al. 2007) adds to the immunological data to indicate that a minimum of five nAChR subtypes (α4β2, α4α5β2, α4α6β2β3, α6β2β3, and α6β2) are expressed in the striatum (Fig. 3).

6.4 Dopamine Release Assays

It is well established that nicotine, and other nicotinic agonists, will elicit Ca^{++}-dependent release of dopamine from striatal tissue slices (see, for examples

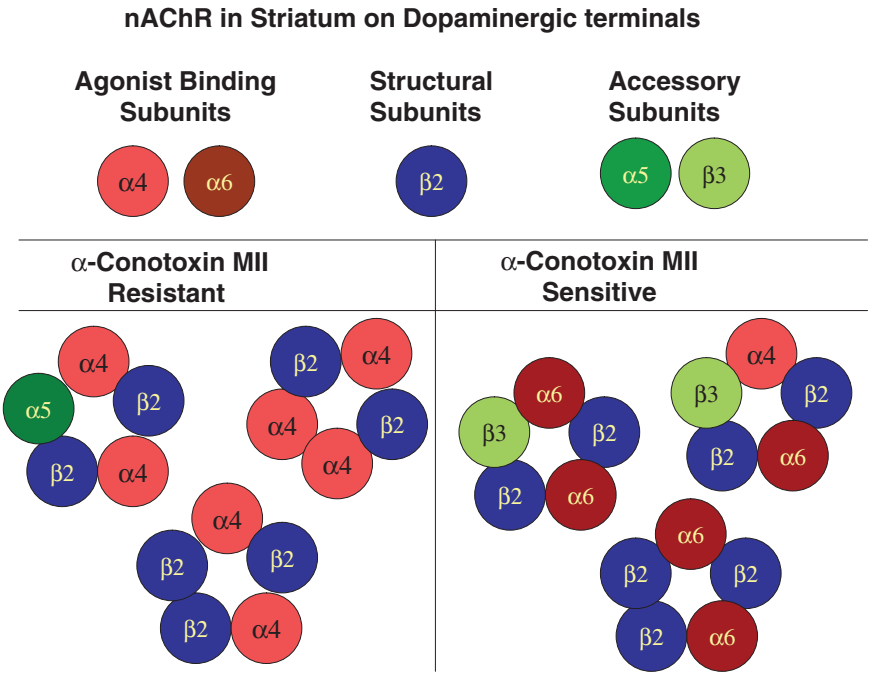

Fig. 3 Potential subunit compositions of nAChRs expressed in dopaminergic nerve terminals. A combination of ligand binding ([^{3}H]-epibatidine and [^{125}I]-α-conotoxin MII), immunoprecipitation, and dopamine release data have led to the conclusion that rodent brain expresses a minimum of five different nAChR subtypes. Three of these (the two forms of α4β2 and α4α5β2) do not bind α-conotoxin MII with high affinity (α-conotoxin MII-resistant). The three α6-containing subtypes bind α-conotoxin MII with high affinity (conotoxin MII-sensitive). In general, the conotoxin-sensitive nAChR subtypes are activated by lower concentrations of agonist than are required to activate the α-conotoxin MII-resistant subtypes (Salminen et al. 2007)

Giorguieff-Chesselet et al. 1979; Dwoskin et al. 1993) and synaptosomes (Rapier et al. 1988). We have used the synaptosomal dopamine release assay in a series of studies that characterized the pharmacological properties of dopamine release from striatum (Cui et al. 2003; Grady et al. 1992, 1994, 1997; Sharples et al. 2000; Whiteaker et al. 2000), and in one study that used the nucleus accumbens, olfactory tubercles, and frontal cortex (Grady et al. 2002). The finding that α-CtxMII is a potent, but partial, inhibitor of nicotinic agonist-stimulated [^{3}H]-dopamine release from mouse (Grady et al. 2002) and rat (Kulak et al. 1997) striatal synaptosomes suggested that more than one nAChR subtype might be expressed on striatal dopaminergic nerve terminals.

Recently, we (Salminen et al. 2004) evaluated the effects of deleting the α2, α4, α5, α7, β2, β3, and β4 genes on both the α-CtxMII-sensitive and resistant components of ACh-stimulated [^{3}H]-dopamine release from striatal synaptosomes. Deletion of the α4 and β2 subunit genes resulted in the total elimination and α5 gene deletion produced a significant decrease in the αCtxMII-resistant

component of ACh-stimulated dopamine release. Deletion of the α2, α7, and β4 did not alter αCtxMII-resistant dopamine release. These results indicate that α4β2 and α4α5β2 nAChRs modulate the αCtxMII-resistant component of dopamine release (Fig. 3).

The α-CtxMII-sensitive component of ACh-stimulated dopamine release is totally absent in striatal synaptosomes obtained from β2 null mutant mice. (Salminen et al. 2004). Identical effects are produced by α6 gene deletion (Champtiaux et al. 2003). Thus, all of the nAChRs that modulate the αCtxMII-sensitive component of dopamine release seem to be α6β2*. Deleting the α4 and β3 genes result in partial reductions in αCtxMII-sensitive release whereas deleting the α2, α7, and β4 genes has no effect (Salminen et al. 2004). These results suggest that dopaminergic nerve terminals express five nAChR subtypes, two that are resistant (α4β2, α4α5β2) and three that are sensitive (α4α6β2β3, α6β2β3, α6β2) to α-CtxMII (Fig. 3). This set of functional subtypes corresponds precisely to those identified with ligand binding and immunoprecipitation (Gotti et al. 2005). Recently, we (Salminen et al. 2007) reported the results of studies that used α4 and β3 null mutant and α4β3 double null mutant mice to evaluate the pharmacological properties of these receptor subtypes. The rank order of EC_{50} values for nicotine-induced dopamine release is: α4α6β2β3 < α6β2β3 ≅ α4(α5)β2 < α6β2.

7 Summary and Conclusions

The discovery that mammalian brain expresses the mRNAs for nine different nAChR subunits (α2–α7, β2–β4) that formed functional receptors when expressed in appropriate combinations in *Xenopus* oocytes suggested that brain tissue might express hundreds of receptor subtypes. This assumes that the brain nAChR(s) are pentameric assemblies that resemble the "peripheral-type" nAChRs that are expressed at the motor endplate or in the electric organs of marine species such as *Torpedo californica* or *Electrophorus electricus*. Fortunately, limited sites of expression and rules of receptor assembly have served to restrict this number enormously. Even so, ongoing research has identified more than ten different nAChR subtypes that differ in many ways. This chapter has summarized only some of the progress that has been made in identifying and characterizing native nAChRs. We have not covered any of the research that has focused on receptors that contain the α2, α3, or β4 subunits because they do not seem to be expressed in high quantities in dopaminergic neurons. We chose to emphasize those neuronal nAChR subtypes that are expressed in dopamine neurons or in neurons that directly interact with dopaminergic neurons in the ventral tegmental area and nucleus accumbens because dopamine seems to be very important in regulating the addiction process. Certainly, the recent discovery that nicotine activates certain α6* nAChRs at lower concentrations than are required for nicotine-induced activation of any of the other known nAChRs, including the nAChR that has been called the high affinity nicotine receptor for nearly 30 years (α4β2*) (Salminen et al. 2007), helps explain why the low doses of nicotine supplied by a single cigarette reinforce tobacco use.

John Langley's early work with nicotine led to the nicotinic receptor concept; he would probably be astonished at how complex the field that he originated has become. It is also likely, however, that he would be delighted to learn that his receptive substance is not a single entity and that nAChRs might play important roles in regulating vital behaviors such as learning and memory as well as psychopathologies such as anxiety, depression, and schizophrenia. Identifying the nAChR subtypes that modulate normal and abnormal behaviors and those that might influence the progression of neurodegenerative diseases could lead to newer and safer therapies.

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Magnetic Resonance Imaging Studies of Cigarette Smoking

Allen Azizian, John Monterosso, Joseph O'Neill, and Edythe D. London

Contents

Abstract This chapter reviews studies that have applied magnetic resonance imaging (MRI) toward a better understanding of the neurobiological correlates and consequences of cigarette smoking and nicotine dependence. The findings demonstrate that smokers differ from nonsmokers in regional brain structure and neurochemistry, as well as in activation in response to smoking-related stimuli and during the execution of cognitive tasks. We also review functional neuroimaging studies on the effects of nicotine administration on brain activity, both at rest and during the execution of cognitive tasks, independent of issues related to nicotine withdrawal and craving. Although chronic cigarette smoking is associated with poor cognitive performance, acute nicotine administration appears to enhance cognitive performance and increase neural efficiency in smokers.

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1 Introduction

Nicotine dependence, usually maintained by cigarette smoking, is a psychiatric disorder that is characterized by compulsive drug-taking and withdrawal upon abrupt cessation of intake (American Psychiatric Association 1994). Although most smokers express a desire to quit, and about one-third of them attempt to do so each year (Centers for Disease Control and Prevention 2002), relapse is common. Only about 14–49% of those who initiate smoking cessation achieve abstinence after receiving nicotine replacement (Silagy et al. 2004), bupropion (Holmes et al. 2004), varenicline (Gonzales et al. 2006), or other combined treatments (Jorenby et al. 2006, 1999; King et al. 2006).

By the mid-1950s, there was sufficient evidence to support the hypothesis of a causal relationship between cigarette smoking and lung cancer; subsequent findings indicated the hazards of smoking to cardiovascular and pulmonary health (for a review see, Kluger 1997). A growing body of evidence, including results of noninvasive brain imaging studies, now suggest that the injurious effects of smoking may extend to the central nervous system. This chapter reviews magnetic resonance imaging (MRI) studies that aimed to clarify the neural correlates of nicotine administration and cigarette smoking. (Related information from nuclear medicine studies appears in the chapter by Sharma and Brody, this volume).

2 Studies of Brain Structure

Medical MRI physics and technology are described in several standard reference works (Kaacke et al. 1999; Weishaupt et al. 2006). Briefly, structural MRI is a noninvasive technique that can be performed repeatedly *in vivo* with minimal risk. To acquire MRI of the brain, the subject is positioned with his head inside a radiofrequency (RF) transmitter coil. Then subject and coil slide into the cylindrical bore of the scanner where a powerful magnetic field is maintained. The field splits the quantum mechanical energy levels of the hydrogen atom nuclei, or "protons," in the brain such that a proton can absorb RF radiation broadcast from the transmitter and thereby be promoted to a higher energy state. After a time delay ("relaxation"), the proton releases the absorbed energy as an electromagnetic disturbance and is registered by a receiver coil that likewise surrounds the subject's head. From the receiver signal, a crisp, 3D picture of the brain composed of 1 mm^3 volume elements ("voxels") is acquired in 5–15 min at clinical field strength (1.5 T) (Jacobs and Fraser 1994). The use of gradients, gradual variations in field strength along the x-, y-, and z-axes of the scanner, enable each voxel to be located in space. The intensity of the MR signal in the voxel is proportional to the density of protons but also varies with the rate of proton relaxation in the voxel. Since these properties vary with tissue type (e.g., gray matter, white matter, CSF), different tissues and different brain structures can be distinguished on the MR image. Advances in MRI have led to new efforts in elucidating the neural basis and sequelae of nicotine dependence (Table 1).

Table 1 Structural MRI studies

Authors	Smokers (*n*)	Nonsmokers (*n*)	FTND	Pack-years	Results
Brody et al. (2004)	19	17	5.1	31	Smokers had smaller gray matter volumes and lower gray matter densities in PFC, smaller volumes left dorsal ACC, and lower gray matter densities in right cerebellum.
Durazzo et al. (2007)	17	36	NR	NA	Among heavy drinkers, smokers had smaller temporal lobe and total gray matter volume than nonsmokers. Heavy drinkers who were smokers also exhibited smaller volumes of temporal, parietal, and total neocortical gray matter.
Gallinat et al. (2006)	22	23	2.9	13.5	Smokers had smaller gray matter volumes & lower gray matter densities in frontal, temporal (including parahippocampal gyrus), and occipital regions than nonsmokers. Group differences (either volume or gray matter density) also found in thalamus, cerebellum, and substantia nigra.
Gazdzinski et al. (2005)	31	36	5.5	26	Chronic smoking in alcohol-dependent individuals and in light drinkers was associated with less parietal and temporal gray matter, and more temporal white matter than in nonsmokers.

FTND Fagerström test for nicotine dependence, *ACC* anterior cingulate cortex; *PFC* prefrontal cortex; *NR* not reported
Data shown for FTND and pack-years are means for the smokers
Pack-years = packs of cigarettes smoked per day × number of years smoked

For example, 1919 participants from the Cardiovascular Health Study (CHS), an ongoing population-based longitudinal study of cardiovascular disease in individuals 65 years old and above, underwent two MRI scans separated by 5 years (Longstreth et al. 2005). The findings indicated a positive correlation of smoking history with sulcal and ventricular expansion (Longstreth et al. 2000, 2001). In a prospective analysis, cigarette smoking also predicted white matter reduction, which was related to vascular disease of the brain and was associated with increased mortality (Longstreth et al. 2005). In another MRI study that evaluated 253 patients over the age of 40, cigarette smoking was positively correlated with the severity grade of periventricular white matter hyperintensities (Fukuda and Kitani 1996) although these findings have not been consistently replicated (Yetkin et al. 1993). Cigarette smoking could injure white matter by increasing blood pressure, reducing oxygen availability, and/or enhancing clotting (Benowitz 2003). Clinical features that have been linked to white matter deficits include impaired cognition and high risk of dementia (Ikram et al. 2007; Kumar and Cook 2002).

In another study, MRI was used to compare 19 smokers with 17 nonsmokers, who were well matched in demographic characteristics and measures of affect (Brody et al. 2004). The smokers had smaller gray matter volumes and densities in dorsolateral and ventrolateral prefrontal cortices (DLPFC, VLPFC), left dorsal anterior cingulate cortex (ACC), and right cerebellum (see Fig. 1). Smoking history (pack-years) also was negatively correlated with prefrontal cortical gray matter density. In line with this study, Gallinat et al. (2006) demonstrated that a group of 22 smokers had smaller gray matter volume than 23 nonsmokers in the frontal lobe, the occipital cortex, the cuneus, and the precuneus. Smokers also had smaller gray matter

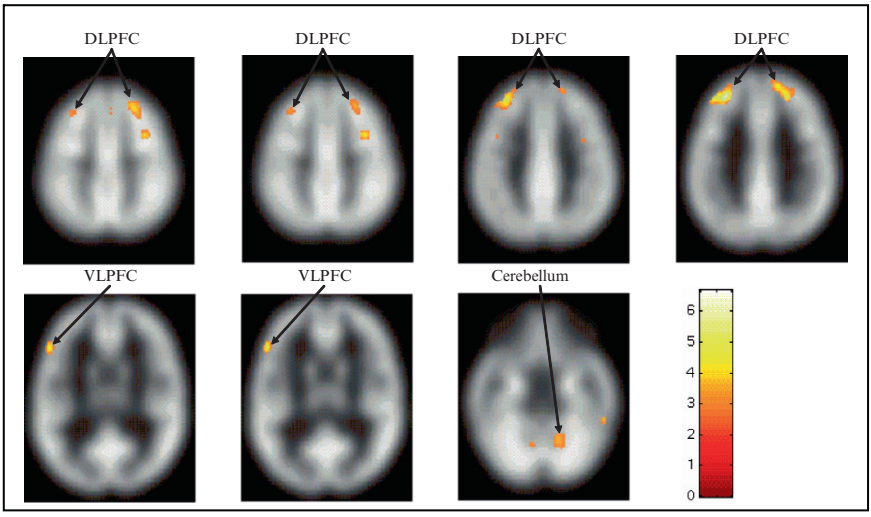

Fig. 1 Voxel-based morphometry showing smaller gray matter volumes and densities in smokers than nonsmokers in dorsolateral and ventrolateral prefrontal cortices (*DLPFC, VLPFC*) and right cerebellum (Brody et al. 2004)

densities in the cerebellum, parts of the temporal and occipital lobes, areas in the middle cingulate cortex, superior frontal gyrus and supplementary motor areas. Consistent with the results of Brody et al. (2004), pack-years of smoking was negatively correlated with gray matter volume in the middle frontal gyrus; the superior, middle, and inferior temporal gyri; the lingual gyrus; and the cerebellum. Neither of the studies indicated higher regional gray matter volumes or densities in smokers than in nonsmokers. While providing consistent findings of gray matter deficits in smokers, these studies lack longitudinal findings or assessments prior to the initiation of smoking. Therefore they do not show whether the group differences reported reflect risk factors, consequences of smoking, or a combination of factors. The negative correlations between pack-years of cigarette smoking and cerebral volume also do not show causality, and as the studies did not adjust for age, age or its interaction with pack-years of smoking could have affected the findings.

Structural deficits such as those described, however, may contribute to the cognitive deficits observed in nicotine-deprived smokers (for review, see Heishman et al. 1994; Parrott et al. 1996). The differences in gray matter volumes and densities in the DLPFC between smokers and nonsmokers are of particular interest. The DLPFC plays an essential role in maintenance and manipulation of information in working memory (Callicott et al. 1999; D'Esposito et al. 1999), and other cognitive domains (Richeson et al. 2003). Nicotine-deprived smokers exhibit performance deficits on tests of working memory (Mendrek et al. 2006), as well as altered activation in the DLPFC associated with working memory (Xu et al. 2005).

Some structural abnormalities that are observed in smokers also occur in individuals with attention deficit hyperactive disorder (ADHD) (Giedd et al. 2001), which is a risk factor for nicotine dependence (Pomerleau et al. 1995). Smoking prevalence rates are approximately 40% vs. 26% for adults (Pomerleau et al. 1995), and 46% vs. 24% for adolescents with ADHD and the general population (Lambert and Hartsough 1998), respectively. Individuals with ADHD initiate cigarette smoking earlier (Milberger et al. 1997) and have more difficulty quitting than smokers without ADHD (23% vs. 51.6% general population) (Pomerleau et al. 1995). Nicotine may enhance attention and alleviate hyperactivity in individuals with ADHD, as do stimulant medications. The high smoking prevalence, therefore, may reflect self-medication (Khantzian 1997). Brain imaging findings indicate that children with ADHD have brain volumes 3–5% smaller than those of age-matched control subjects (Castellanos et al. 2002), with differences being most prominent in the DLPFC, the caudate nucleus, the pallidum, the corpus callosum, and the cerebellum (for reviews see Giedd et al. 2001; Seidman et al. 2005). These deficits in the prefrontal cortex and cerebellum (and perhaps other structures) are also seen in nicotine dependence.

Cigarette smoking also is commonly linked to alcoholism, with ~80% of alcohol-dependent individuals using tobacco products (Miller and Gold 1998) and 50–90% of individuals seeking treatment for alcohol-use disorder showing comorbidity for nicotine dependence (Daeppen et al. 2000). In one study of brain morphology in 1-week abstinent, alcohol-dependent individuals (Gazdzinski et al. 2005), the participants were retrospectively grouped as current smokers ($n = 24$) or

nonsmokers ($n = 13$). Thirty healthy light drinkers (23 nonsmokers, seven smokers) served as comparison subjects. The results indicated that alcohol dependence and cigarette smoking had independent effects on brain morphology, and the combination of the two diagnoses was associated with greater deficits in parietal gray matter volume than either factor alone. Alcohol dependence, irrespective of smoking status, was associated with smaller volumes of frontal and parietal white matter, parietal and temporal gray matter, and thalamus, and with widespread sulcal enlargements. In line with the previous findings, irrespective of alcohol consumption, smokers had smaller parietal and temporal gray matter, and greater white matter than nonsmokers. More recently, volumetric comparisons were made among age-matched smoking heavy drinkers ($n = 17$), nonsmoking heavy drinkers ($n = 16$), and nonsmoking light drinkers ($n = 20$) (Durazzo et al. 2007). Smoking heavy drinkers demonstrated smaller temporal lobe and global gray matter volumes than nonsmoking heavy drinkers. Nonsmoking heavy and light drinkers did not differ significantly on gray matter volumes. Taken together, these studies provide evidence that alcohol dependence and cigarette smoking are associated with independent and additive effects in the brain.

In conclusion, structural MRI studies demonstrate anatomical deficits in the brains of cigarette smokers. The extent to which these deficits preexisted smoking and to which smoking cessation can reverse these deficits is unknown. However, some commonalities in brain structural findings with respect to ADHD and nicotine dependence suggest a common etiology. In addition, studies of the comorbidity of alcohol dependence with nicotine dependence indicate independent effects, illustrating that cigarette smoking may explain some of the variance reflected in the morphological abnormalities of alcohol dependence and potentially other conditions.

3 Proton Magnetic Resonance Spectroscopy (^{1}H MRS)

^{1}H MRS is another noninvasive technique performed in conventional MRI scanners. "Single-voxel" MRS typically samples the brain with spatial resolution of $\sim$2 cc and scan time $\sim$5 min. Magnetic resonance spectroscopic imaging (MRSI) acquires data from $\sim$4–1000 voxels simultaneously at $\sim$1 cc resolution and scan time $\geqslant$8 min. While structural MRI principally measures the concentration of water protons in each voxel, proton MRS yields voxel concentrations of less abundant hydrogen proton-bearing compounds ("metabolites"). (MRS can also be performed with MR-active nuclei other than the proton, but discussion of those techniques exceeds the scope of this review.) Due to differences in the local chemical environment, each metabolite absorbs RF radiation from the MR transmitter at a slightly different frequency, measured in parts per million (ppm) of the scanner field frequency. An MR spectrum is then reconstructed out of the receiver signal manifesting as a series of "peaks" or "resonances" each of which identifies a metabolite (or family of metabolites with a common functional group) in the sample. The area under the peak is proportional to the concentration of the metabolite in the voxel with adjustments for relaxation rates.

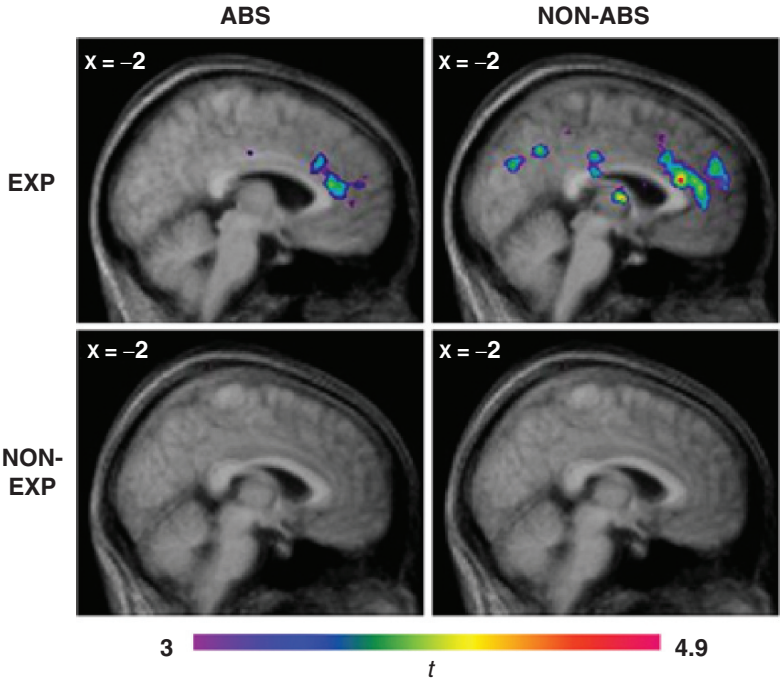

Fig. 2 Neural activation in responses to smoking (vs. neutral) cues, showing the effects of expectancy to smoke and by abstinence states. Overall, the network of brain regions recruited by smoking-related cues was affected only slightly by abstinence, but was affected dramatically by expectancy, with greater activation observed when participants believed they would be allowed to smoke after the scan (*top*) (McBride et al. 2006)

At 1.5 T, several metabolites are readily quantified in human brain with proton MRS (Birken and Oldendorf 1989; Maier 1995; Fig. 2). After the water peak, the largest resonance (2.01 ppm) is assigned to *N*-acetyl-aspartate (NAA), the second most abundant amino acid in the brain. The likely main function of NAA is to transport excess water, including water generated by glucose catabolism, out of neurons (Baslow 2003). Overlapping NAA is a small peak for *N*-acetyl-aspartyl-glutamate (NAAG), which appears to serve as a storage form for glutamate (Glu) as well as a post-synaptic NMDA receptor antagonist. A shoulder to NAA at 2.1–2.5 ppm is formed by the sum of Glu and glutamine (Gln) – denoted "Glx." Glu is the major excitatory neurotransmitter of the CNS and the most abundant amino acid in the human brain; Gln is another probable storage form of Glu. At 3.01 ppm there is a major peak representing the sum of creatine and phosphocreatine, denoted "Cr." Cr may also reflect the energy state of brain tissue because creatine and phosphocreatine maintain an ATP "buffer" for short-term cell energy demands (Erecinska and Silver 1989; Miller 1991). Phosphocreatine is also thought to serve as a key brain osmolyte (Miller et al. 2000; Ross and Bluml 2001). Export or import of phosphocreatine and other osmolytes (including NAA, Glu, choline compounds [Cho], and inositol

compounds [mI]) also helps regulate cell water content. The Cho peak represents multiple compounds, including phosphocholine, glycerophosphocholine, choline proper, and acetylcholine, active in membrane metabolism (Stork and Renshaw 2005). Finally, inositol compounds are chiefly represented by the cyclic sugar alcohol *myo*-inositol. A precursor to membrane phosphatidylinositol, mI is a substrate for the phosphoinositide second-messenger system; therefore, changes in mI levels may reflect abnormalities in membrane metabolism and/or intracellular signaling (Stork and Renshaw 2005). In addition, mI is produced as a product of glucose metabolism, linking it to cell energetics (Cecil et al. 2006).

MRS metabolites also reflect the relative densities and/or metabolic activities of neurons and glia in brain tissue. In particular, NAA and NAAG are abundant in neurons, but nearly absent from mature glia (Simmons et al. 1991; Urenjak et al. 1993), and Cr, Cho, and mI, though present in both neurons and glia, are more abundant in glia (Urenjak et al. 1993; Brand et al. 1993). Assessment of metabolite abnormalities provides information that might predict susceptibility to or consequences of cigarette smoking. The concurrent analyses of these markers in the context of anatomical abnormalities may provide clues regarding the biochemical basis of functional deficits identified in nicotine-dependent individuals.

In the MRS studies reviewed here, smokers were allowed to smoke *ad libitum* to prevent nicotine withdrawal during the assessment (Table 2). The initial MRS studies focused on the potential effects of chronic cigarette smoking on neurochemical markers in alcohol-dependent individuals. In one study, 24 recovering alcohol-dependent individuals, abstinent 1 week (14 smokers, ten nonsmokers), and 26 light-drinking comparison subjects (seven smokers, 19 nonsmokers) were compared in the MRS assessment of gray and white matter of neocortical lobes, basal ganglia, midbrain, and cerebellar vermis (Durazzo et al. 2004). As with the studies of brain structures reviewed above, the combined effects of alcohol dependence and chronic smoking were associated with greater adverse effects than either factor alone. Irrespective of cigarette smoking, alcohol dependence was associated with lower frontal NAA and Cho, and with lower parietal and thalamic Cho. In contrast, cigarette smoking, independent of alcohol consumption, was associated with lower midbrain NAA and Cho, as well as lower cerebellar vermis Cho. Among smoking alcoholics, the severity of nicotine dependence and number of cigarettes smoked per day were negatively correlated with thalamic and lenticular NAA. In the same group, lower NAA in the cerebellar vermis was associated with slower perceptual-motor speed; however, among nonsmoking alcoholics, lower NAA in the vermis was correlated with deficits in learning and memory performance. The results suggest additive effects of cigarette smoking and alcoholism on metabolites, especially NAA, in several brain regions, notably the frontal lobes and the cerebellum. These brain regions are involved in higher-order cognitive processes, as well as fine and gross motor functions.

The thalamus has one of the highest densities of nicotinic acetylcholine receptors in the brain and is thought to be a critical structure in nicotine dependence (Clarke 2004; Rubboli et al. 1994a, b). As mentioned, Durazzo et al. (2004) obtained

Table 2 Magnetic Resonance Spectroscopy Studies

Authors	Smokers (*n*)	Nonsmokers (*n*)	Ex-smokers (*n*)	FTND	Pack-years	Results
Gallinat et al. (2007)	13	13	NA	NR	15.3	NAA in left hippocampus but not ACC was lower in smokers than nonsmokers. Cho in ACC was positively correlated with pack-years.
Galliant and Schubert (2007)	13	16	9	NR	15.1	Glu in hippocampus or ACC did not differ between groups. In current smokers, number of cigarettes per day, pack-years, age of smoking initiation did not correlate with Glu.
Durazzo et al. (2004)	21	29	NA	6	25	Among recovering alcoholics, smokers had lower frontal white matter NAA and lower midbrain Cho than nonsmokers. Severity of nicotine dependence and number of cigarettes per day were negatively correlated with thalamic and lenticular NAA. Smoking also was associated with lower midbrain NAA and Cho and with lower Cho in cerebellar vermis.
Durazzo et al. (2006)	14	11	NA	NR	NR	Over 1 month of abstinence from alcohol, recovering alcoholics showed significant increases of NAA and Cho in frontal and parietal lobes. These increases appeared to be driven by data from nonsmokers.
Mason et al. (2006)	12	8	NA	4.5	NR	Among alcohol-dependent subjects, cortical GABA was greater in nonsmokers than in smokers at 1 week but not at 1 month of sobriety. Irrespective of alcohol dependence, smokers had higher levels of Glu and Gln than nonsmokers.

FTND Fagerström test for nicotine dependence; *NR* not reported; *NA* not applicable; *NAA* *N*-acetyl-aspartate; *Glu* glutamate; *Gln* glutamine; *Cho* choline; *ACC* anterior cingulate cortex; *PFC* prefrontal cortices

Data shown for FTND and pack-years are means for the smokers

Pack-years = packs of cigarettes smoked per day × number of years smoked

negative correlations between severity of nicotine dependence and number of cigarettes smoked per day with thalamic NAA, and further noted a positive association between duration of smoking and thalamic Cr. A possible mechanism leading to these thalamic results follows from evidence that local application of nicotine to the thalamus increases turnover of dopamine and norepinephrine in rats (Kubo et al. 1989). Conceivably, after repeated exposure, the thalamus downregulates monoamine oxidase (MAO), which catalyzes catabolism of biogenic amines, including dopamine, norepinephrine, and serotonin, producing ammonia and water (Cooper et al. 1986). In fact, laboratory studies of rats and PET studies of humans demonstrate that cigarette smoke decreases levels of both the MAO_A and MAO_B variants of the enzyme (reviewed in Volkow et al. 1999). Both MAO_A and MAO_B are present in neurons and glia. All things being equal, a reduction of MAO activity consequently diminishes ammonia and increases water content in both cell types. Biosynthesis of Gln from Glu in glia requires ammonia (Kvamme et al. 1985), while biosynthesis of Glu from Gln in neurons yields ammonia (Martinez-Hernandez et al. 1977). Therefore, a drop in MAO activity should lead to increased Glu in both cell types. In glia, excess Glu may be removed through the Krebs cycle (Petroff et al. 2000). Ultimately, this leads to higher ATP production. We suggest that chronically higher ATP leads to an expansion of the creatine–phosphocreatine pool that buffers ATP and, hence to a larger MRS Cr signal with more years of smoking, as seen by Durazzo et al. (2004).

In neurons, two additional pathways are available for Glu disposal, one being through vesicular export at remote synapses. Similar to the action of mesopontine acetylcholine (Kobayashi and Isa 2002), nicotine can sensitize thalamic relay neurons by slightly depolarizing their phospholipid membrane, making them more likely to discharge in response to sensory stimuli. Therefore, synaptic export of Glu is likely to be enhanced in smokers. The second neuronal mechanism for Glu reduction is capture by NAA (Cangro et al. 1987). Since NAAG often colocalizes with Glu in synaptic vesicles (Neale et al. 2000), discharge also releases NAAG (Williamson and Neale 1988). This two-step process would reduce thalamic NAA in smokers, as reported by Durazzo et al. (2004). Shuttling of Glu into the Krebs cycle and consequent rise in Cr could occur in neurons as well as glia. The overall mechanism proposed here could be tested by 3-T short-TE MRS studies of the thalamus, which would look for diminished Gln/Glu and elevated NAAG/NAA ratios in smokers. Similar effects might apply in other brain regions.

MRS has also been used to measure effects of alcohol abuse and smoking on NAA, Glu, and GABA in the occipital cortex (Mason et al. 2006). The occipital cortex was selected because of its sensitivity to alcohol and for greater ease of MRS acquisition. Twelve alcohol-dependent men (seven smokers, five nonsmokers) were tested twice in the first month of sobriety (after $\sim$1 week and 1 month), and compared to eight healthy men (five smokers, three nonsmokers) who were scanned once. In the initial scan, alcohol-dependent smokers exhibited lower GABA than alcohol-dependent nonsmokers. At the second scan, alcohol-dependent nonsmokers, but not alcohol-dependent smokers, exhibited an abstinence-related decrease in GABA, rendering

the two groups not significantly different at the end of 1 month. In addition, irrespective of alcohol dependence, smoking was associated with higher Glu. The results suggest that smoking prevents alcohol withdrawal changes in cortical GABA, which might have important implications for detoxification medicine.

The first study to examine metabolite concentrations in nicotine-dependent individuals without alcohol dependence measured absolute levels of NAA, Cho, and Cr in the left hippocampus and the ACC (Gallinat et al. 2007). The results indicated that NAA was lower in nicotine-dependent individuals ($n = 13$) than in nonsmokers ($n = 13$) in the left hippocampus but not in the ACC, with no group differences in Cho or Cr concentrations in either area. ACC Cho was positively correlated with smoking history. According to the authors, nicotine-deprivation among smokers is associated with deficits in working memory, and the hippocampal NAA alterations might lead to memory dysfunction. The contribution of the hippocampus in working memory, however, is questionable and further research in support of this hypothesis is needed. Since Cho is higher in glial cells than in neurons (Brand et al. 1993; Urenjak et al. 1993), the positive correlation between ACC Cho concentration and smoking history might suggest local pathological sequelae, such as microglial proliferation, due to heavy smoking. A second study assessed Glu in 13 current chronic smokers, nine former smokers, and 16 nonsmokers in the left hippocampus and ACC (Gallinat and Schubert 2007). The result did not support significant group differences in Glu in either region. Moreover, no significant correlations between Glu and age of smoking onset, cigarettes per day, or smoking history emerged. The results imply that Glu in left hippocampus and ACC is not affected by smoking and that further research in other regions is needed.

In summary, *in vivo* proton MRS findings indicate that cigarette smoking and alcohol abuse are accompanied by abnormal brain metabolism in some areas of the brain. Cigarette smoking may have effects that are independent of alcohol consumption, and the two forms of drug abuse may have additive effects on metabolite deficits in the frontal lobes and the cerebellum. Other evidence indicates low NAA in the left hippocampus of nicotine-dependent individuals without alcohol dependence. The results highlight the need to consider the possible contributions of both alcohol abuse and cigarette smoking when investigating the brain correlates of either. Combining MRS with structural studies of the brain may help determine the effects of cigarette smoking on metabolites that occur alongside of or independently from regional volumetric or morphometric changes. It is possible that molecular alterations detected by MRS predate the appearance of the gross abnormalities revealed in anatomical studies, but it is unclear whether metabolic alterations represent risk indicators for nicotine dependence or reflect the adverse effects of cigarette smoking. Follow-up studies are needed to examine the predictive value of MRS measurements and to determine whether they normalize after smoking cessation, as is seen for MAO_B levels with PET (Fowler et al. 1996). Future research is also needed to determine if metabolic abnormalities are related to the cognitive deficits that smokers experience after abrupt abstinence.

4 Functional Magnetic Resonance Imaging

Blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI) provides a noninvasive tool for mapping activation in the human brain with a spatial resolution of 2–3 mm, and a temporal resolution of 1–4 s. The basic principle of BOLD functional MRI is that increased neuronal activity is associated with increased blood flow in the surrounding region. Because the increased perfusion delivers oxygen that exceeds the increase in metabolic demand, the ratio of oxygenated to deoxygenated hemoglobin increases. As hemoglobin is diamagnetic when oxygenated but paramagnetic when deoxygenated, the increase in the ratio of oxy- to deoxyhemoglobin produces local increases in MR signal that reflect the increase in neuronal activity.

Functional MRI has enabled researchers to begin to characterize how nicotine and smoking affect neural activity, how cues associated with smoking affect the brain, and how acute and chronic smoking affect brain functioning during the execution of specific cognitive tasks. Throughout the review of this work, which follows, it is important to bear in mind that the effects of cigarette smoking are not limited to those associated with nicotine, but can also include those of thousands of constituents of tobacco smoke and of nonchemical (i.e., behavioral) factors.

In the first fMRI study on the effect of acute nicotine administration on participants tested at rest (i.e., not engaged in a cognitive probe task), Stein et al. (1998) administered nicotine (0.75, 1.50, and 2.25 mg/70 kg body weight) intravenously to 16 active smokers. Dose- and time-dependent BOLD signal increases occurred in several cortical and subcortical regions, with prominent signal changes in the cingulate, dorsolateral, and medial frontal regions (Stein et al. 1998). Findings in these regions were consistent with the cortical distribution of radiolabeled nicotine accumulation in brain as mapped with PET (Nyback et al. 1989), suggesting that the observed fMRI signal changes reflected alterations in neuronal activity secondary to CNS nicotinic receptor activation. Consistent with findings from earlier self-administration experiments (e.g., Henningfield et al. 1983) dose-dependent increases in drug liking and feelings of euphoria (“high”) were also noted. A large correspondence was observed in the time course of subjective and physiological measures with peaks in plasma nicotine, fMRI signal increases, and subjective effects all occurring 2–3 min postinjection. In addition to concluding that nicotine is critically involved in the reinforcing effects of smoking, the authors argued that the robust increases in frontal and cingulate activation occasioned by nicotine suggest that behavioral and cognitive effects of smoking result directly from the neuropharmacological sequelae of nicotine, rather than from nonspecific effects of alleviating withdrawal symptoms (Stein et al. 1998).

5 Neural Responses to Stimuli Associated with Smoking

Environmental cues associated with drug intake play a substantial role in the maintenance of habits and contribute to relapse (Abrams et al. 1988). The traditional model of addiction emphasizes affect, whereby drug-related cues trigger feelings of

euphoria or of withdrawal, both of which can lead to increased drug-seeking motivation (O'Brien et al. 1986). In a nicotine-dependent individual the sight or smell of a burning cigarette can lead to drug craving (Carter and Tiffany 1999). Localization of the neural substrates of this conditioned response can lead to development of new treatments geared towards interrupting or minimizing the effects of cue-induced craving for cigarettes. Many functional imaging studies have examined the effects of smoke-related stimuli on brain activation (Table 3).

Due et al. (2002) tested 12 nicotine-deprived smokers (10 h) and six matched nonsmokers with fMRI on an oddball target detection task in which participants were required to press a button only when a picture belonging to the target category (animals) appeared. Targets were intermixed within more frequent distracter pictures that had either smoking content (45%) or neither smoking nor animal content. The smoking-related stimuli were pictures of people smoking or holding a cigarette. The neutral images were matched in content but did not contain smoking-related items. When the fMRI signal during presentation of neutral stimuli was subtracted from the fMRI signal during presentation of stimuli with smoking-related content, the difference was greater in nicotine-dependent individuals than in nonsmoker comparison subjects in prefrontal cortex (inferior frontal gyrus, middle frontal gyrus) and in a network of subcortical regions (posterior amygdala, posterior hippocampus, ventral tegmental area, and medial thalamus). According to the authors, the effect in the prefrontal cortex suggests that smoke-related nontargets were processed as if they were target stimuli, and they elicited a pattern of activation that was associated with allocation of visuospatial attention. The activation in mesocorticolimbic circuits was consistent with the evocation of emotional and appetitive responses by smoking-related cues. The authors interpreted the activation in these circuits to indicate that smoke-related images acquire high motivational salience and mediate reinforcement even in the absence of nicotine itself.

In another study, in which 14 overnight abstinent smokers and 12 nonsmokers viewed smoking-related and neutral pictures during fMRI, the smoking-related images elicited greater activation in the ventral striatum and nucleus accumbens in smokers than in nonsmokers (David et al. 2005). In addition, smoking-related (vs. neutral) images produced bilateral activation in the ACC, orbitofrontal cortex, superior frontal gyrus, and occipital cortex in smokers. Taken together, these studies indicate that stimuli associated with cigarette smoking activate a network of frontal regions and subcortical brain regions that have been implicated in craving for other drugs of abuse (Bonson et al. 2002; Childress et al. 1999; Kilts et al. 2004).

Both the perceived availability of drugs and the duration of abstinence can affect cue-elicited neural activity as well (Wilson et al. 2005). In one study, 19 nicotine-dependent individuals were exposed to smoking-related and smoking-unrelated videos while during conditions of varied expectancy (participants were either allowed to smoke immediately or 4 h after the scan) and varied level of withdrawal (abstinence for previous 12 h or previous *ad libitum* smoking) were varied (McBride et al. 2006). While brain activation by smoking-related cues was affected only slightly by the withdrawal manipulation, the effect of expectancy was dramatic, with greater activation when participants expected to smoke after the scan. Significant

Table 3 Brain fMRI responses to stimuli associated with smoking

Authors	Smokers (*n*)	Nonsmokers (*n*)	FTND	Pack-years	Results
Brody et al. (2007)	42	NA	5.7	24.5	Attempts to suppress craving were associated with activation in the left dorsal ACC, PCC, and precuneus, and reduced activity in the cuneus bilaterally, left lateral occipital gyrus, and right postcentral gyrus.
David et al. (2005)	14	12	4.7	NR	Smoking-related images elicited greater activation in ventral striatum and nucleus accumbens in smokers than nonsmokers. Among smokers, smoking-related cues produced bilateral activation in the ACC, OFC, SFG, and occipital cortex.
Due et al. (2002)	12	6	NR	NR	Smoking-related stimuli produced greater activation in nicotine-dependent subjects than in nonsmokers in prefrontal cortex, hippocampus, amygdala, ventral tegmental area, and thalamus.
Franklin et al. (2007)	21	NA	4.8	NR	Smoking-related cues elicited bilateral activation in the amygdala, ventral striatum, thalamus, hippocampus, insula, and OFC. Perfusion in DLPFC and PCC was positively correlated with craving.
McBride et al. (2006)	20	NA	<5	NR	Recruitment of brain regions by smoking-related cues was affected only slightly by a withdrawal manipulation but dramatically by an expectancy manipulation (greater activation when participants believed they would be allowed to smoke after the scan). Significant cue-induced activation of prefrontal, associative and paralimbic regions was observed only in subjects expecting to smoke after the scan.
McClernon et al. (2007b)	20	NA	6.5	20	Exposure to smoking cues produced greater activation of right superior frontal gyrus and right insula in DRD4 L compared to DRD4 S individuals. In contrast, exposure to smoking cues among DRD4 S individuals resulted in no significant increase in activation compared to DRD4 L individuals.

McClernon et al. (2007a)	20	NA	⩽5	20	Responses to smoking-related cues in the amygdala were attenuated following 2–4 week of smoking cessation treatment. A similar pattern was observed in the thalamus among 1-month abstinent, but not relapsing participants.
Smolka et al. (2006)	10	NA	3	8.3	Severity of nicotine dependence was positively correlated with fMRI signal change in response to smoking cues in brain areas related to visuospatial attention (dorsal ACC, inferior parietal cortex, secondary visual cortex, parahippocampal and fusiform gyri) and in brain regions involved in motor preparation and imagery.
Wilson et al. (2005)	20	NA	NR	NR	Visual, posterior parietal, and temporal cortices showed differential activation during presentation of smoking-related vs. neutral stimuli independent of whether or not participants expected to smoke. Subregions of the prefrontal cortex (ventromedial, ventrolateral, and dorsolateral prefrontal cortices) showed cue-elicited activation that was modulated by smoking expectancy.

FTND Fagerström test for nicotine dependence, *M* mean; *NR* not reported; *NA* not applicable; *ACC* anterior cingulate cortex; *PCC* posterior cingulate cortex; *OFC* orbitofrontal cortex; *SFG* superior frontal gyrus; *DLPFC* dorsal lateral prefrontal cortex; *DRD4L* dopamine receptor 4 variable number tandem repeat
Data shown for FTND and pack-years are means for the smokers
Pack-years = packs of cigarettes smoked per day × number of years smoked

cue-induced activation of the prefrontal, associative and paralimbic regions occurred only in participants expecting to smoke after the scan (see Fig. 2). Because self-reported craving in response to the cues was, by contrast, not significantly affected by expectation, the findings suggested that prefrontal, associative and paralimbic response to cues may have less to do with craving than with regulation and planning of drug-seeking behavior. These findings also have implications for study design in that not only the time of abstinence before scanning but also the anticipated time when the subject will have access to the drug postscanning can affect results.

In another study, arterial spin-labeled (ASL) perfusion MRI was used to investigate cue-induced craving (Franklin et al. 2007). ASL uses radiofrequency pulses to label arterial blood (water) magnetically to detect modulations in cerebral blood flow. Unlike BOLD-based fMRI, ASL perfusion fMRI provides an absolute measure of blood flow (Detre et al. 1992). While the BOLD method relies on contrasting alternating event or block types within scan sessions, the perfusion method allows contrasting signal from a single condition in one test session (e.g., during a session-long craving induction) with signal from a signal condition in a second test session (e.g., during a session of viewing neutral stimuli). This feature may be particularly advantageous for investigating the neural correlates of craving, since craving may persist, making it difficult to modulate in the "on–off" fashion that is optimal for BOLD fMRI. The comparison of one craving induction session with one neutral session that is possible with ASL has been done in PET studies of craving (Bonson et al. 2002; Brody et al. 2002; Childress et al. 1999). In these PET studies, smoking-related cues elicited more activity than the neutral stimuli in the amygdala, ventral striatum, thalamus, hippocampus, insula, and orbitofrontal cortex. Moreover, perfusion in DLPFC and posterior cingulate correlated positively with subjective craving.

The repeated observation of insular recruitment in fMRI studies of cue reactivity, replicating findings with cerebral glucose metabolism (see Brody et al. 2002 and also the chapter by Sharma and Brody, this volume), is remarkable in the light of recent findings from a brain lesion study (Naqvi et al. 2007). That study found that a greater proportion of smokers who suffered lesions involving the insula, compared with those who had damage to other brain areas, exhibited "disrupted smoking," quitting immediately after the brain damage without any difficulty or persistent craving (odds ratio $= 22.05$, $p = 0.005$). Current evidence suggests that the insula plays a role in conscious feelings by anticipating the bodily effects of emotional events (Damasio et al. 2000), and this finding suggests that such anticipation contributes to cigarette craving.

The extent to which cigarette craving is an issue in maintaining smoking behavior may depend on a person's level of nicotine dependence. This question was addressed in a test of the relationship between severity of nicotine dependence (measured by the Fagerström test for nicotine dependence, FTND; Heatherton et al. 1991) and cue-elicited neural activation (Smolka et al. 2006). Ten nondeprived smokers participated in a cue reactivity fMRI-probe task in which they were instructed to observe visual stimuli that either did or did not contain smoking-cues. The results revealed that the severity of nicotine dependence was positively correlated with fMRI signal change in response to smoking cues in brain areas related to visuospatial atten-

tion (dorsal ACC, inferior parietal cortex, secondary visual cortex, parahippocampal and fusiform gyri) and in brain regions involved in motor preparation and imagery (premotor cortex, supplementary motor areas, as well as left primary motor cortex). As the authors pointed out, the premotor area corresponds to the site of mirror neurons that discharge not only in response to execution of action, but also during the observation of others carrying out actions (Smolka et al. 2006). The intensity of cue-induced craving was independently correlated with BOLD activation in brain regions implicated in goal-directed behaviors (amygdala, substantia nigra, tegmental pedunculopontine nucleus) and others related to episodic memory (hippocampus, parahippocampal gyrus, inferior temporal gyrus, middle temporal and fusiform gyrus, medial occipital lobe and gyrus, and the cerebellum).

In addition to psychological factors that contribute to individual differences in nicotine dependence, identification of genetic markers has been a topic of recent research efforts. In a recent fMRI study of response to smoking cues, McClernon et al. (2007b) considered the association between smoking cue response and the dopamine receptor 4 variable number tandem repeat (DRD4 VNTR) polymorphism, which codes the dopamine 4 (D4) receptor. The DRD4 VNTR polymorphism occurs within a proline-rich coding region, and the 7-repeat (long) variant (DRD4 L) appears to blunt the intracellular response to dopamine in vitro, as compared with the 2- and 4-repeat (short) variants (DRD4 S) (Asghari et al. 1995). DRD4 L has also previously been associated with higher novelty seeking (Ebstein 2006) as well as with greater subjective response to smoking cues among cigarette smokers (Hutchison et al. 2002) and greater response to heroin cues among heroin addicts (Shao et al. 2006). In the McClernon et al. (2007b) fMRI study, exposure to smoking cues resulted in greater activation of right superior frontal gyrus and right insula in DRD4 L compared to DRD4 S individuals. In contrast, exposure to smoking cues among DRD4 S individuals resulted in no significant increase in activation compared to DRD4 L individuals. These findings are interesting in the light of a study examining the relationship between candidate genes regulating brain dopamine transmission and dopamine release stimulated by smoking as measured by PET (Brody et al. 2006). In that study, individuals with fewer than seven repeats of the DRD4 VNTR (DRD4 S) exhibited significantly greater smoking-induced dopamine release in brain-reward areas (ventral caudate/nucleus accumbens) than individuals with seven or more repeats (DRD4 L). This finding, when considered alongside previous self-report (Hutchison et al. 2002) and current fMRI findings, suggests that DRD4 L individuals have less dopaminergic response to smoking but greater self-reported craving and brain activation in response to environmental cues.

Given that environmental cues trigger craving and can cause relapse, a major goal of treatment must be to alleviate craving responses to drug cues. McClernon et al. (2007a) investigated the neural responses to smoke-related cues before and after an extinction-based treatment. In this treatment, smokers switch to low nicotine cigarettes prior to quitting (Rose et al. 2006). Nicotine-dependent individuals were scanned in a cue-reactivity task at baseline, following 2–4 weeks of smoking reduced nicotine content cigarettes while wearing a 21-mg nicotine patch, and 2–4 weeks following smoking cessation. Results revealed that extinction-based

treatment can modulate brain responses to conditioned smoking cues. Relative to the baseline phase, neural responses to smoke-related cues in the amygdala were attenuated following 2–4 weeks of smoking cessation treatment. Moreover, a similar pattern was observed in the thalamus among 1-month abstinents, but not relapsing participants. The results provide preliminary evidence that effectiveness of extinction might be operationalized as a decrease in brain activation in areas that trigger drug-seeking behavior.

Brody and colleagues (2007) reported on the use of fMRI in the investigation of neural substrates suppressing cigarette craving. In their study, smokers underwent fMRI while (i) viewing neutral video clips, (ii) viewing smoking video clips without attempting to suppress their craving, or (iii) viewing smoking video clips while trying to suppress their craving. Relative to viewing of the cues without suppression, attempts at craving suppression were associated with increased activation in the left dorsal ACC, posterior cingulate cortex (PCC), and precuneus, and with decreased activation in the cuneus bilaterally, left lateral occipital gyrus, and right postcentral gyrus. The reported dorsal ACC activation and visual cortical deactivation are consistent with examinations of brain function during cognitive reappraisal and cognitive modulation of emotion (Kalisch et al. 2006; Ochsner et al. 2004). Engagement of the ACC, which is implicated in conflict avoidance and attentional control (Barch et al. 2001; Braver et al. 2001), was hypothesized to reflect the active direction of attention away from the hyper-salient smoking stimuli as an effortful process that is contrary to automatic patterns of attention. The authors suggested that actively suppressing the urge to smoke involves a redistribution of resources from sensory and motor areas to limbic (and related) brain areas.

Taken together, these findings demonstrate that fMRI has been a useful tool for studying the neural correlates of cue-induced craving. They have shown that: (i) nicotine-dependent individuals exhibit more activation that nonsmokers in brain regions linked to attention and motivation in response to smoking-related cues; (ii) intensity of craving is positively correlated with brain activation in orbitofrontal cortex, DLPFC, and cingulate gyrus; (iii) contextual factors, such as availability of cigarettes, can affect neural activity; and (iv) individual differences in severity of nicotine dependence and in genotype can modulate cue-induced reactivity. New techniques including perfusion MRI may well allow additional progress in this area, possibly leading to clinical applications of fMRI.

6 Cognitive Effects Related to Smoking

More than three decades of research indicates that smoking has both acute and chronic effects on cognition (Belanger et al. 2007); and fMRI provides a powerful tool for investigating neural correlates of these deficits. Perhaps because difficulty in concentrating is part of nicotine withdrawal (American Psychiatric Association 1994) and a likely barrier to success in smoking cessation attempts, most fMRI work has focused on cognitive domains generally classified as “executive functions,” including sustained attention and working memory (Table 4).

Table 4 Neural activation associated with sustained attention and working memory

Authors	Smokers (*n*)	Nonsmokers (*n*)	FTND	Pack-year	Cognitive test	Results
Lawrence et al. (2002)	15	14	4.5	4.7	RVIP	In smokers, nicotine improved task performance and increased neural activity in the parietal and occipital cortices, the thalamus and caudate, and decreased activity in left frontal, anterior and posterior cingulate, insula, and left parahippocampal regions.
Xu et al. (2007)	13	9	4.9	15.3	Stroop	The differences in BOLD signal changes between Stroop conditions showed a group x test interaction in the right percentral sulcus, including the putative right frontal eye field. Smokers, but not nonsmokers, showed greater changes (relative to rest) in BOLD signal in the incongruent than in the congruent condition in the first fMRI test (before smoking a cigrette) but not in the second test (after smoking).
Xu et al. (2005)	8	NA	4.1	14.4	N-Back	Task-related neural activity in the left DLPFC showed a significant interaction between test session & working memory load. When smokers had smoked *ad libitum*, task-related activity in the left dorsolateral prefrontal cortex was relatively low for an easy task condition (1-back), and increased as task difficulty increased; but when smokers were abstinent overnight, activity in the L-DLPFC was approximately as high at low task level as it was at more difficult levels.

FTND Fagerström test for nicotine dependence; *RVIP* rapid visual information processing;
NA not applicable; *DLPFC* dorsal lateral prefrontal cortex
Data shown for FTND and pack-years are means for the smokers
Pack-years = packs of cigarettes smoked per day × number of years smoked

7 Sustained Attention and Working Memory

Lawrence and colleagues (2002) explored the neural substrates of nicotine effects on sustained attention using the rapid visual information processing (RVIP) task. Smokers ($n = 15$) received either placebo or 21-mg transdermal nicotine patch prior to testing. Matched nonsmokers ($n = 14$) were tested under similar conditions, but did not receive a nicotine patch. Relative to the placebo condition, the smokers in the nicotine condition demonstrated improved task performance and increased neural activity in the parietal and occipital cortices, the thalamus and caudate, and decreased activity in left frontal, anterior and posterior cingulate, insula, and left parahippocampal regions (see Fig. 3). As noted by the authors, previous studies

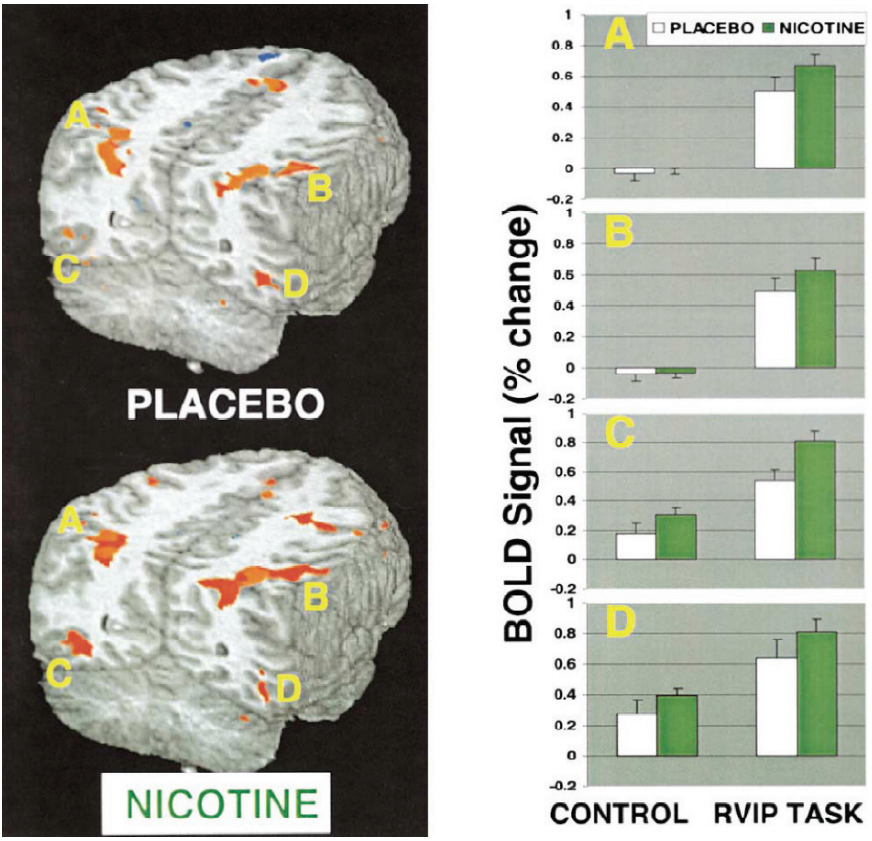

Fig. 3 Activation difference between the rapid visual information processing (RVIP) and control tasks in smokers after administration of placebo and nicotine patches. The graphs show the percent change in activation (from baseline) during performance of each task in smokers in the two drug conditions. After receiving nicotine, smokers demonstrated better task performance and more task-related activity in the parietal (*A* and *B*) and occipital (*C* and *D*) cortices compared to the parallel measures after they received placebo (Kumari et al. 2003)

point out that thalamus and caudate contain high densities of nicotinic acetylcholine receptors, and increased neural activity in these regions may reflect modulations in arousal and motor activity that improves sustained attention.

While the report by Lawrence et al. (2002) assessed the effects of nicotine administration, cigarette smoke includes other chemicals, and smoking includes psychological reinforcers that extend beyond nicotine or the chemical constituents of cigarette smoke. In a recent study, we tested 13 nonsmokers and nine nicotine-dependent smokers during fMRI while they performed the Stroop color-word naming task (Xu et al. 2007). The Stroop task requires participants to indicate the color in which a word is presented. In the "congruent" condition, the word is consistent with its presentation color (e.g., "BLUE" in blue text) while in the incongruent condition, the word is inconsistent with its presentation color (e.g., "RED" displayed in blue text). In Xu et al. (2007), research participants were scanned in two tests, separated by a 15-min break. Smokers were allowed to smoke *ad libitum* up to 45–60 min before the first test. After acquisition of the first set of fMRI, each participant was removed from the scanner for 15 min. Smokers smoked a cigarette of their usual brand, and nonsmokers took a break but did not smoke. The differences in BOLD signal changes between Stroop conditions (incongruent minus congruent) showed a group x-test interaction in the right precentral sulcus, including the putative right frontal eye field (FEF). Smokers, but not nonsmokers, showed greater changes (relative to rest) in BOLD signal in the incongruent than in the congruent condition in the first fMRI test but not in the second (see Fig. 4). The results suggest that even after a brief abstinence from smoking, nicotine-dependent individuals exhibit compromised functional efficiency in the right FEF and adjacent precentral sulcus in a test of selective attention, and that smoking alleviates this condition. Studies indicate that in chronic smokers, abstinence has deleterious effects on working memory, and

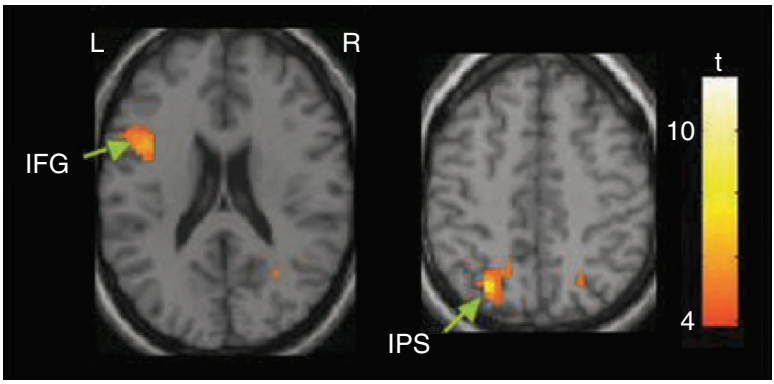

Fig. 4 Smokers (but not nonsmokers) showed greater changes in BOLD signal in the left inferior frontal gyrus (*IFG*) and intraparietal sulcus (*IPS*) from the rest condition in the incongruent than in the congruent condition. These changes were pronounced in the first fMRI test (45–60 min after the last cigarette of *ad libitum* smoking) but not in the second (<20 min of *ad libitum* smoking) (Xu et al. 2007)

resumption of smoking alleviates this withdrawal-induced deficit (Mendrek et al. 2006; Tait et al. 2000). Studies using parametric versions of working memory tests, such as the N-back task, have demonstrated load-sensitive fMRI signal in lateral prefrontal, parietal, and medial supplementary motor cortices in healthy research participants (Carlson et al. 1998; Jansma et al. 2000). Our research group investigated brain activity in eight cigarette smokers while they performed the N-back test under relative satiety (<1.5 h abstinence) and overnight abstinence (>14 h) sessions (Xu et al. 2005). Task-related neural activity in the left DLPFC showed a significant interaction between test session and N-back working memory load (1-back, 2-back, 3-back). When smokers had smoked *ad libitum*, task-related activity in the left DLPFC cortex was relatively low for an easy task condition (1-back), and increased as task difficulty increased; but when smokers were abstinent overnight, activity in the left DLPFC was approximately as high at low task level as it was at more difficult levels. These results were consistent with earlier findings using PET (Bonson et al. 2002) and were hypothesized to mean that neural processing related to working memory in the left DLPFC was less efficient following overnight abstinence relative to satiety. Taken together, cognitive testing studies (both in and out of the scanner) suggest that "executive function" measures are sensitive to the difficulty in concentration that is experienced by smokers during acute withdrawal. The neuroimaging studies that have reported neural correlates of effects on these tasks, not surprisingly, have prominently (though certainly not exclusively) implicated the frontal and parietal cortices. It bears repeating that cross-sectional fMRI data are correlational, and so these studies do not allow us to conclude that these regions are causally relevant in the observed effects of acute abstinence and acute smoking on cognition. Longitudinal studies may more easily allow such inferences.

8 Effects of Nicotine in Nonsmokers

Most studies investigating the effects of nicotine on cognition have involved smokers, and interpretation of the findings is limited as results could reflect withdrawal effects or preexisting deficits that predispose individuals to nicotine dependence. One way to avoid these potentially confounding effects in studies of response to nicotine per se is to test nonsmokers (Table 5). In such a study of 12 healthy nonsmokers, subcutaneous administration of nicotine (12 mg) improved accuracy in all conditions of the N-back task, and improved response times during the more demanding conditions of the task (Kumari et al. 2003). Irrespective of the drug condition (nicotine or placebo), frontal and parietal regions showed task-related activity that varied with increasing task difficulty; but nicotine increased task-related activity in the anterior cingulate (0-back, 1-back, and 2-back), superior frontal (1-back and 2-back), and left superior parietal cortices (1-back, 2-back, and 3-back). Moreover, in the 3-back condition, task-related activity was lower in the right superior parietal cortex after nicotine than after placebo administration. The authors concluded that greater task-related activity after nicotine than after placebo administration may reflect the mediating effect of nicotine on attention and arousal systems (Kumari et al. 2003).

Table 5 Brain fMRI responses to effects of nicotine in nonsmokers

Authors	Nonsmokers (*n*)	Nicotine administration	Cognitive test	Results
Giessing et al. (2006)	15	Nicorette polacrilex gum, 1 and 2 mg	Cued target detection	Nicotine did not affect performance, but modulated activity in the right IPS depending on whether cues were valid or invalid. Nicotine reduced signal change in the invalid trials and enhanced signal change during the valid trials in the context of low cue reliability.
Kumari et al. (2003)	12	2 mg nicotine, subcutaneously	N-Back	Nicotine improved accuracy in all N-back conditions and reduced response times during the more demanding conditions of the task. Irrespective of drug condition, frontal and parietal regions were activated with increasing memory load. Nicotine, however, increased brain activation in the anterior cingulate (0-back, 1-back, 2-back), superior frontal (1-back, 2-back), and left superior parietal cortex (1-back, 2-back, 3-back). In the 3-back condition, nicotine reduced activation in the right superior parietal cortex.
Thiel et al. (2005)	15	Nicorette polacrilex gum, 1 and 2 mg	Cued target detection	Nicotine reduced neural activity in the left IPS and precuneus, and decreased RTs to invalid cues. Only participants who were slow in the placebo session showed benefits. The alerting-related nicotine effects were associated with reduction of activation in the right angular and middle frontal gyri.

(continued)

Table 5 (continued)

Vossel et al. (2007)	24	Nicorette polacrilex gum, 1 mg	Cued target detection	Distributional analysis of reaction times revealed that nicotine decreased the validity effect more in the high than in the low validity cue condition. Nicotine reduced orienting-related activation in the right parietal brain regions (TPJ). This effect occurred only in the condition with the high valid cues. Conversely the low valid cue condition increased neural activation in the right parietal regions.
Thiel and Fink (2007)	16	Nicorette polacrilex gum, 2 mg	Cued target detection	Nicotine modulated alertness-brain related activity in several brain regions, but did not significantly influence response times. In the visual condition, nicotine decreased alertness-brain activity in the right lateral posterior superior temporal gyrus. In the auditory modality, effects of nicotine were trial-specific: decreased activation in frontal and occipitoparietal regions in warned trials and increased activation in unwarned trials.

IPS intraparietal sulcus; *TPJ* temporoparietal junction.

Other research has investigated the neural networks that underlie alertness and the effect of nicotine on activity in these networks. In a study of 15 nonsmokers who received nicotine and placebo before fMRI paired with a test of visual–spatial attention, nicotine reduced neural activity in the left intraparietal sulcus and precuneus and improved response times to reorientation of attention after invalid cues (Thiel et al. 2005). Only those research participants who were slow in the placebo session showed the performance benefits. The alerting-related nicotine effects were associated with reduction of neural activity in the right angular gyrus and the middle frontal gyrus. In a separate study, the same authors tested the effects of nicotine in 24 nonsmokers who completed a target-detection task (Vossel et al. 2007). The participants were cued as to where the target would appear before its presentation. In the "high validity cue" condition, the cue was valid in 90% of the trials, while in the "low validity cue" condition, the cue was valid in 60% of the trials. Distributional analysis of response times revealed that nicotine decreased the validity effect more in the high validity cue than in the low validity cue condition. In line with the previous finding, nicotine reduced orienting-related neural activity in right parietal brain regions (superior parietal cortex, inferior parietal sulcus, temporoparietal junction) during the high validity cues. Conversely, the low validity cue condition increased neural activity in right parietal regions (superior parietal cortex/inferior parietal sulcus, angular gyrus).

The effects of nicotine on task-related activity in 15 nonsmokers was investigated in another study involving the detection of valid and invalid cued targets in the context of changing cue reliability (Giessing et al. 2006). While nicotine did not affect behavioral performance, nicotine reduced the difference in BOLD signal between invalid and valid trials in the right intraparietal sulcus. Effects on changes in the BOLD signal, but not on response time, suggest that neuroimaging is a sensitive tool that may measure subtle drug effects that modulate cognitive strategies but not behavioral responses. The results of this study also support the notion that the parietal cortex is part of the neural network involved in visuospatial attention and is sensitive to both nicotine and cue evaluation.

In another study, the modality-specific mechanisms that underlie alertness were investigated (Thiel and Fink 2007). Nonsmokers ($n = 16$) were tested under placebo and nicotine conditions in a target-detection task employing visual and auditory stimuli. Irrespective of stimulus modality, nicotine modulated alertness-related brain activity in several regions. In line with the previous work, nicotine did not significantly influence response times. In the visual condition, nicotine decreased alertness-brain activity in the right lateral posterior superior temporal gyrus. In contrast, nicotine effects in the auditory modality were trial-specific and manifested in decreased activity for the warned trials and increased activity for the unwarned trials in occipitoparietal and frontal regions. The authors concluded that the effects of nicotine on brain mechanisms that underlie alertness are selective to stimulus modality and stimulus type.

MRI has contributed a wealth of information regarding the neurobiological correlates and consequences of cigarette smoking and nicotine dependence. Recent findings demonstrate that smokers differ from nonsmokers in regional brain structure

and neurochemistry, as well as in activation in response to smoking-related stimuli and during the execution of cognitive tasks. Advances in brain imaging technology continue to enhance our understanding of the neurobiological correlates and consequences of cigarette smoking. Among promising developments in this area are novel methods that are currently in use in several laboratories that allow for neuroimaging simultaneous to naturalistic smoking (Frederick et al. 2007). This approach may lead to a better understanding of the neural sequelae of cigarette smoking, of the neural substrates of the desire to smoke, and of the motivations engaged during voluntary restraint from smoking.

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In vivo Brain Imaging of Human Exposure to Nicotine and Tobacco

Anil Sharma and Arthur L. Brody

Contents

Abstract While most cigarette smokers endorse a desire to quit smoking, only 14–49% will achieve abstinence after 6 months or more of treatment. A greater understanding of the effects of smoking on brain function may result in improved pharmacological and behavioral interventions for this condition. Research groups have examined the effects of acute and chronic nicotine/cigarette exposure on brain activity using functional imaging; the purpose of this chapter is to synthesize findings from such studies and present a coherent model of brain function in smokers. Responses to acute administration of nicotine/smoking include reduced global brain activity; activation of the prefrontal cortex, thalamus, and visual system; activation of the thalamus and visual cortex during visual cognitive tasks; and

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increased dopamine (DA) concentration in the ventral striatum/nucleus accumbens. Responses to chronic nicotine/cigarette exposure include decreased monoamine oxidase (MAO) A and B activity in the basal ganglia and a reduction in $\alpha_4\beta_2$ nicotinic acetylcholine receptor (nAChR) availability in the thalamus and putamen (accompanied by an overall upregulation of these receptors). These findings indicate that smoking enhances neurotransmission through cortico–basal ganglia–thalamic circuits by direct stimulation of nAChRs, indirect stimulation via DA release or MAO inhibition, or a combination of these and possibly other factors. Activation of this circuitry may be responsible for the effects of smoking seen in tobacco-dependent smokers, such as improvements in attentional performance, mood, anxiety, and irritability.

1 Introduction

Smoking remains a major health issue in USA and quitting smoking continues to be a challenge. In a recent survey, approximately 23% of Americans were found to smoke cigarettes (Balluz et al. 2004). While most smokers endorse a desire to quit (Fiore et al. 2000), very few will quit smoking without treatment, and only about 14–49% will achieve abstinence after 6 months or more of effective treatment (Holmes et al. 2004; Hughes et al. 1999; Hurt et al. 1997; Jorenby et al. 1999; Killen et al. 2000, 1999). Because cigarette smoking carries both considerable health risks (Bartal 2001; Mokdad et al. 2004) and high societal costs (Leistikow et al. 2000a, b), there is an urgent need for improved treatments for this condition. Functional brain imaging (in conjunction with other lines of research) holds great promise for elucidating both brain circuits and molecular targets that mediate the acute effects of cigarette smoking and the chronic effects of tobacco dependence. A greater understanding of brain function associated with smoking may result in improved pharmacological (and behavioral) interventions.

Many functional brain imaging studies of tobacco use and dependence have been performed, using four primary imaging modalities: (i) functional magnetic resonance imaging (fMRI), (ii) positron emission tomography (PET), (iii) single photon emission computed tomography (SPECT), and (iv) autoradiography. These imaging modalities have been used to determine relationships between brain function and the effects of acute and chronic cigarette smoking and of smoking-related behaviors. For this chapter, the MEDLINE database was searched using keywords for the four imaging techniques mentioned above, cross-referenced with the words “nicotine”, “cigarette”, and “tobacco.” Only data-driven functional imaging studies were included in this review, and reference lists within papers found on MEDLINE were also examined and relevant studies included here. In order to maintain focus in this chapter, functional imaging techniques that provide measures of blood flow and metabolism (which are closely related under normal conditions; Paulson 2002) are combined under the general heading of brain activity (including fMRI and certain types of SPECT, PET, and autoradiography studies). Also, in order to build

a cohesive model of brain activity responses to acute and chronic smoking, nicotine and cigarette studies will be reviewed together while recognizing that cigarette smoke has many constituents other than nicotine (Baker et al. 2004; Fowles and Dybing 2003).

The purpose of this chapter is to synthesize findings from functional brain imaging studies of tobacco use and dependence, and present a coherent model of brain function in smokers. Acute brain responses to nicotine/smoking will be reviewed first, followed by chronic responses to nicotine/smoking, and concluding with a discussion of these imaging findings in the context of neuroanatomical work and the clinical effects of smoking in tobacco-dependent subjects.

2 Brain Function Responses to Acute Nicotine Administration and Cigarette Smoking

2.1 Brain Activity Responses to Nicotine/Cigarette Administration

Many functional brain imaging studies have been performed examining the effects of administration of nicotine or cigarette smoking compared with a placebo or control state (Table 1). Though a wide range of brain regions have been reported to have altered activity in response to nicotine or cigarette smoking, several global and regional findings have been replicated, leading to general conclusions about the acute effects of nicotine or smoking on brain activity.

One common finding is that nicotine administration (Domino et al. 2000b; Stapleton et al. 2003b) or cigarette smoking (Yamamoto et al. 2003) results in decreased global brain activity. Similarly, smokers who smoke ad lib prior to SPECT scanning (including the morning of the scan) have decreased global brain activity compared to former smokers and nonsmokers (Rourke et al. 1997). These findings are generally supported by studies using transcranial Doppler ultrasound or the Xe 133 inhalation method to measure responses to smoking, with some (Cruickshank et al. 1989; Kubota et al. 1983, 1987; Rogers et al. 1983), but not all (Kodaira et al. 1993; Terborg et al. 2002), studies showing diminished cerebral blood flow.

A large ($n = 86$), recent study (Fallon et al. 2004) further characterized this decreased global activity with nicotine administration. ^{18}F-fluorodeoxyglucose (FDG) PET was performed while smokers and exsmokers performed the Bushman aggression task (designed to elicit an aggressive state) and wearing either a 0, 3.5-, or 21-mg nicotine patch. Smokers who were rated high on the personality trait hostility had widespread cerebral metabolic decreases while wearing the 21-mg patch and performing the aggression task. Low-hostility smokers did not have these changes during PET, suggesting that personality profile may determine which smokers have global metabolic decreases in response to nicotine.

In studies examining regional activity responses to nicotine or smoking, the most common findings are relative increases in activity in the prefrontal cortex (including the dorsolateral prefrontal cortex, and inferior frontal, medial frontal, and

Table 1 Functional brain imaging studies of nicotine or cigarette administration

Authors	Subjects	Method	Intervention	Results
Animal studies				
London et al. (1988a, b)	Rats	2-Deoxy-D-[1–^{14}C]glucose autoradiography	SC nic (0.1–1.75 mg kg^{-1})	↑ Nicotine rich regions, including thal, cereb, visual system, others
Marenco et al. (2000)	Rats – chronically nic exposed vs. nic naive	2-Deoxy-D-[1–^{14}C] glucose autoradiography	SC nic (0.4 mg kg^{-1}) vs. saline	↑ Thal, superior colliculus in chronically exposed; ↑ thal, superior colliculus, medial habenula, and dorsal lateral geniculate in nic naive
Human studies				
Rourke et al. (1997)	8 Smokers; 8 former smokers; 17 nonsmokers	Iodine-123 iodoamphetamine (IMP) SPECT	Smokers smoked the morning of the scan; other groups did not	↓ Cortical uptake of IMP (a measure of blood flow) in current smokers compared to other groups
Stein et al. (1998)	16 Smokers	fMRI	IV nic (0.75–2.25 mg/70 kg wt) vs. placebo	↑ R NAc and bilateral amyg, cingulate, frontal lobes, thal, others
Domino et al. (2000a)	18 Smokers	^{15}O-PET	Nic nasal spray vs. pepper spray	↑ Thal, pons, visual cortex, cereb
Domino et al. (2000b)	11 Smokers	FDG-PET	Nic nasal spray vs. pepper spray	Small ↓ global; ↑ L IFG, L PC, R thal, visual cortex; ↓ normalized L ins and R inf occ ctx

Zubieta et al. (2001)	18 Smokers	^{15}O-PET	Nic nasal spray vs. pepper spray	↑ Anterior thal; ↓ L ant temp and R amyg
Rose et al. (2003)	34 Smokers	^{15}O-PET	Cigarette vs. no nic control conditions	↑ L frontal factor (incl. prefrontal and ACC), ↓ L amyg rCBF
Yamamoto et al. (2003)	10 Smokers	99mTc-ECD SPECT	Cigarette vs. abstinence	↓ Global blood flow
Stapleton et al. (2003a)	4 Smokers; two nonsmokers	2 FDG-PETs (fully quantified)	IV nic (1.5 mg) vs. placebo	↓ Global and most regions studied
Zubieta et al. (2005)	19 smokers	^{15}O-PET	Nicotine containing vs. denicotinized cigarettes	↓ Global blood flow
Staley et al. (2006)	16 Smokers; 16 nonsmokers	5 IA-SPECT	Recent abstinence	↑ Striatum, parietal cortex, frontal cortex, anterior cingulated, temporal cortex, occipital cortex, cerebellum

All regional changes represent normalized activity, unless otherwise stated. *SC* subcutaneous, *nic* nicotine, *thal* thalamus, *cereb* cerebellum, *SPECT* single photon emission computed tomography, *fMRI* functional magnetic resonance imaging, *IV* intravenous, *R* right, *L* left, *NAc* nucleus accumbens, *amyg* amygdala, *FDG* ^{18}F-fluorodeoxyglucose, *PET* positron emission tomography, *IFG* inferior frontal gyrus, *PC* posterior cingulate, *ins* insula, *inf occ ctx* inferior occipital cortex, *ant* anterior, *temp* temporal lobe, *ACC* anterior cingulate cortex

orbitofrontal gyri) (Domino et al. 2000b; Rose et al. 2003; Stein et al. 1998), thalamus (Domino et al. 2000a, b; London et al. 1988a, b; Stein et al. 1998; Zubieta et al. 2001), and visual system (Domino et al. 2000a, b; London et al. 1988a, b). Additionally, a Xe 133 inhalation study reported increases in frontal lobe and thalamic blood flow in smokers who smoked a cigarette (Nakamura et al. 2000). The human studies here examined cigarette smokers, while the animal studies here used non-dependent rats, with strong concordance of findings between these sets of studies. Functional brain imaging studies of nicotine or cigarette administration to human nonsmokers have not yet been reported, and would be important for a more complete understanding of the effects of tobacco on brain activity. While this group of studies demonstrates specific regional activation with nicotine or smoking, they also imply activation of cortico–basal ganglia–thalamic brain circuits (Alexander et al. 1990) that mediate the subjective effects of smoking (see Sect. 4). Zubieta et al. (2005) have conducted a ^{15}O-PET study in 19 smokers using nicotine and denicotinized cigarettes, who were abstinent of smoking for 12 h before PET. In this study, increases in the regional cerebral blood flow (rCBF) in visual cortex and cerebellum, and reductions in rCBF in the anterior cingulate, the right hippocampus, and ventral striatum were found. Cigarette craving in chronic smokers also was correlated with rCBF in the right hippocampus, which is a region involved in associating environmental cues with drugs, and in the left dorsal anterior cingulate, an area implicated in drug craving and relapse to drug-seeking behavior.

Since regional activity was normalized to whole brain activity in at least some of these studies, and whole brain activity has been found to decrease with nicotine or cigarette administration, the regional findings presented here may represent either increased regional activity or, possibly, less of a decrease in regional activity than in other brain areas. Regional decreases in activity are generally not seen with nicotine or cigarette administration, though at least two studies found relatively decreased activity in the amygdala, left (Rose et al. 2003) and right (Zubieta et al. 2001)).

2.2 Effect of Nicotine on Brain Activation During Cognitive Tasks

There is evidence that nicotine administration improves performance on tasks that require vigilant attention in nicotine-dependent smokers (Newhouse et al. 2004). Nicotine administration also has been reported to improve reaction time, regardless of smoking status (Ernst et al. 2001a). Consistent with these findings are studies that demonstrate that acute abstinence from smoking (within 12 h) results in slowed response times (Bell et al. 1999; Gross et al. 1993; Thompson et al. 2002).

In examining brain mediation of the cognitive effects of smoking, several groups have performed functional imaging studies in subjects performing cognitive tasks during administration of nicotine (compared to a control condition) (Table 2). For most of these studies, subjects performed a cognitive task that involved visual recognition and working memory, such as the n-back task. Results of these studies have been somewhat mixed, showing both decreased (Ernst et al. 2001b;

Table 2 Functional brain imaging studies of nicotine or cigarette administration during cognitive tasks/stimulation

Authors	Subjects	Method/task	Intervention	Effect of nicotine during task
Ghatan et al. (1998)	12 Smokers; 6 nonsmokers	^{15}O-butanol PET/computerized maze	IV nic infusion versus abstinence	↓ ACC and cerebellum; ↑ occ ctx
Ernst et al. (2001b)	11 Smokers; 11 former smokers	^{15}O-PET/2-back	Two pieces of 2-mg nic gum vs. placebo gum	↓ ACC and PFC activation in smokers
Jacobsen et al. (2002)	9 Smokers	fMRI/photic stimulation	IV nic 10 mcg kg^{-1} vs. saline	No effect on visual cortex
Lawrence et al. (2002)	15 Smokers	fMRI/rapid visual information-processing	21-mg nic vs. placebo patch	↑ Parietal and occipital ctx,, thal, caudate
Kumari et al. (2003)	11 Nonsmoking men	fMRI/n-back	SC nic (1 mg) vs. saline	↑ ACC, superior frontal ctx, superior parietal ctx
Jacobsen et al. (2004)	13 Schizophrenic smokers; 13 smokers	fMRI/n-back	28- or 35-mg nic vs. placebo patch	↑ ACC and bilateral thal activation (schizophrenic, nonschizophrenic)

PET positron emission tomography, *IV* intravenous, *nic* nicotine, *ACC* anterior cingulate cortex, *occ ctx* occipital cortex, *PFC* prefrontal cortex, *thal* thalamus, *fMRI* functional magnetic resonance imaging

Ghatan et al. 1998) and increased (Jacobsen et al. 2004; Kumari et al. 2003) anterior cingulate cortex (ACC) activation in response to nicotine administration while performing the task. Brain activation responses to nicotine during cognitive tasks have been more consistent in other brain areas such as the thalamus (Jacobsen et al. 2004; Lawrence et al. 2002) and visual cortex (Ghatan et al. 1998; Lawrence et al. 2002), while nicotine had no effect on the visual cortex during photic stimulation (Jacobsen et al. 2002). This last finding indicates that nicotine activates the visual cortex only during demanding visual tasks, rather than on simple stimulation.

2.3 Brain Dopamine Responses to Nicotine and Smoking

A common pathway for the positive reinforcement associated with most, if not all, addictive drugs is the brain dopamine (DA) reward pathway (Koob 1992; Leshner and Koob 1999). Laboratory animal studies demonstrate that DA release in the ventral striatum (VST)/nucleus accumbens (NAc) underlies the reinforcing properties of nicotine (Koob 1992; Leshner and Koob 1999). Microdialysis (Damsma et al. 1989; Di Chiara and Imperato 1988; Pontieri et al. 1996; Sziraki et al. 2001) and lesion (Corrigall et al. 1992) studies in rats indicate that nicotine-induced DA release is strongest in this region, and is more robust than the DA release found in associated structures receiving dopaminergic input, such as the dorsal striatum (Di Chiara and Imperato 1988). These studies generally used nicotine dosages that simulated human cigarette smoking. Acute exposure to cigarette smoke and nicotine has been found to upregulate dopamine transporter mRNA in the ventral tegmental area (VTA) and substantia nigra (Li et al. 2004), and chronic exposure to cigarette smoke, more so than chronic nicotine alone, has also been found to upregulate D_1 and D_2 receptor mRNA in the VST (Bahk et al. 2002). Additionally, many in vitro studies of the VST have reported DA release in response to nicotine administration (Connelly and Littleton 1983; Marien et al. 1983; Rowell et al. 1987; Sakurai et al. 1982; Westfall et al. 1983).

Functional brain imaging studies of the DA system (Table 3) corroborate and expand upon these laboratory findings. Striatal DA release in response to a nicotine or cigarette challenge has been demonstrated repeatedly in both nonhuman primates and humans (Brody et al. 2004b, 2006; Dewey et al. 1999; Marenco et al. 2004; Tsukada et al. 2002), with most of these studies using PET and the radiotracer ^{11}C-raclopride (a specific D_2/D_3 DA receptor binder) to demonstrate DA release through radiotracer displacement. These studies have reported a wide range of DA concentration change. In two studies that examined the question directly (Marenco et al. 2004; Tsukada et al. 2002), nicotine was found to result in less radiotracer displacement than amphetamine, while it has also been reported that nicotine-induced DA release is comparable in magnitude to that induced by other addictive drugs (Pontieri et al. 1996). Also, an association between ^{11}C-raclopride displacement and the hedonic effects of smoking (defined as elation and euphoria) has been demonstrated (Barrett et al. 2004), though this study did not find an overall difference between the

Table 3 Functional imaging studies of the effects of nicotine or cigarette smoking on the dopamine (DA) system

Authors	Subjects	Method	Intervention	Results/conclusions
Dewey et al. (1999)	16 Baboons	^{11}C-Raclopride PET (double bolus)	IV nic (0.3 mg)	↓ DV tracer (indicating ↑ DA concentration) in NAc
Dagher et al. (2001)	11 Smokers; 18 nonsmokers	^{11}C-SCH 23390 PET		↓ BP in smokers (indicating ↓ D1 receptor density) in ventral striatum
Tsukada et al. (2002)	4 Macada mulatto monkeys	^{11}C-Raclopride PET (B/I)	IV nic (B/I)	Slight ↓ BP (indicating ↑ DA concentration) in anesthetized, but not conscious monkeys, in dorsal striatum
Salokangas et al. (2000)	9 Smokers; 10 nonsmokers	^{18}F-DOPA PET		↑ Uptake (indicating ↑ DA activity) in cd and Put of smokers
Krause et al. (2002)	11 Smokers w/ADHD; 11 nonsmokers w/ADHD	[^{99m}Tc]TRODAT SPECT		↓ DAT (striatal) in smokers
Staley et al. (2001)	21 Smokers; 21 nonsmokers	[^{123}I] β-CIT SPECT		No overall binding difference between smokers and nonsmokers; ↑ brainstem 5-HT transporters in male smokers
Marenco et al. (2004)	5 Rhesus monkeys	^{11}C-Raclopride PET (double bolus and B/I)	IV nic (0.01–0.06 mg kg^{-1})	↓ BP (indicating ↑ DA concentration) in basal ganglia with nic administration
Brody et al. (2004b)	20 Smokers	^{11}C-Raclopride PET (B/I)	Single cigarette vs. no smoking	↓ BP (indicating ↑ DA concentration) in smoking, but not no smoking, condition in L ventral cd and put
Barrett et al. (2004)	10 Smokers	^{11}C-Raclopride PET (double bolus)	Smoking every 12 min vs. no smoking	↓ BP correlated with hedonic response to smoking in cd and posterior put

PET positron emission tomography, *IV* intravenous, *nic* nicotine, *DV* volume of distribution, *DA* dopamine, *BP* binding potential, *B/I* bolus-plus-infusion, *cd* caudate, *put* putamen, *SPECT* single photon emission computed tomography, *DAT* dopamine transporter, *ADHD* attention deficit hyperactivity disorder, *β-CIT* 2 β-carbomethoxy-3 β-(4-iodophenyl)-tropane, *5-HT* serotonin

smoking and nonsmoking conditions. Thus, while most studies do provide evidence for nicotine/smoking-induced DA release, there are disparities between studies in the extent of human smoking-induced DA release, leaving this issue currently unresolved. Disparities between these studies may be due to differences in methodology (e.g., nicotine administration vs. cigarette smoking) and/or technical complexities in performing such studies. (As an aside, effects of smoking on dopamine projections to the prefrontal cortex (Goldman-Rakic et al. 1989) have not yet been reported with functional brain imaging.)

Nicotine-induced DA release in the NAc has been reported to be mediated by stimulation of nicotinic acetylcholine receptors (nAChRs) on cells of the VTA that project to the NAc rather than by nicotinic receptors within the NAc itself (Nisell et al. 1994). Lesioning of mesolimbic VTA neurons projecting to the NAc leads to decreased nicotine self-administration (Corrigall et al. 1992; Lanca et al. 2000). Additionally, the effects of nicotine on the dopaminergic system appear to be modulated by glutamatergic and GABAergic neurons (Picciotto and Corrigall 2002), with nicotine stimulation of gluatamatergic tracts from the prefrontal cortex to the VTA leading to increased DA neuron firing (Kenny and Markou 2001) and GABA agonism leading to a dampening of DA neuron responses (Cousins et al. 2002). Recent work indicates that nicotine administration causes prolonged depression of GABAergic firing, leading to relatively large excitatory (glutamatergic) input into the mesolimbic DA system and increased DA neuron firing (Mansvelder et al. 2002).

Other functional imaging studies of the DA system have reported decreased D_1 receptor density (Dagher et al. 2001), increased ^{18}F-DOPA uptake (a marker for increased DA turnover) (Salokangas et al. 2000), and both decreased (Krause et al. 2002) and no alterations (Staley et al. 2001) in dopamine transporter binding in smokers.

To summarize these studies of the DA system, there is extensive evidence that nicotine administration and smoking result in activation of the brain DA mesolimbic pathway, resulting in increased DA release and turnover in the VST/NAc. Because dopaminergic input to the NAc modulates neurotransmission through cortico–basal ganglia–thalamic circuitry (Haber and Fudge 1997), smoking-induced increases in DA concentration may explain some of the clinical effects of smoking, as discussed in Sect. 4.

2.4 Functional Imaging of Nicotinic Acetylcholine Receptors (nAChRs)

Because stimulation of nAChRs is intimately linked with the effects of smoking, a longstanding and still developing area of research is the labeling of nAChRs using functional brain imaging. Nicotinic acetylcholine receptors are ligand-gated ion channels consisting of α and β subunits (Court et al. 2000; Hogg et al. 2003). Many nAChRs have been identified, with the heteromeric $\alpha_4\beta_2$ being the most common subtype in the brain and the homomeric α_7 being the next most common.

Postmortem (Benwell et al. 1988; Breese et al. 1997) and laboratory (Yates et al. 1995) studies demonstrate that smokers have widespread upregulation of nAChRs, likely related to desensitization of these receptors from nicotine exposure. Many animal studies also demonstrate upregulation of nAChRs in response to chronic nicotine administration (e.g., Pauly et al. 1996; Shoaib et al. 1997; Zhang et al. 2002). Thus, nAChRs are a natural target for tracer development in the pursuit of a greater understanding of tobacco dependence and other illnesses with abnormal nAChR levels.

Animal research demonstrates that nicotine binds to nAChRs in the brain to mediate a variety of behavioral states (Lukas 1998), such as heightened arousal and improved reaction time and psychomotor function (Paterson and Nordberg 2000). Nicotine administration also produces reward through DA release in the NAc, at least in part through stimulation of nAChRs in the VTA (Blaha et al. 1996; Corrigall et al. 1994; Nisell et al. 1994; Yeomans and Baptista 1997; Yoshida et al. 1993). Nicotinic acetylcholine receptors are widespread throughout the brain, with a rank order distribution of nAChR density being thalamus > basal ganglia > cerebral cortex > hippocampus > cerebellum (Broussolle et al. 1989; Cimino et al. 1992; Clarke et al. 1984; Davila-Garcia et al. 1999, 1997; London et al. 1985, 1995; Pabreza et al. 1991; Pauly et al. 1989; Perry and Kellar 1995; Valette et al. 1998; Villemagne et al. 1997).

Radiotracers for the nAChR have been developed in recent years, with labeled A-85380 (3-(2(*S*)-azetidinylmethoxy pyridine) (Koren et al. 1998) compounds having the most widespread use. Radiolabeling of A-85380 was a major advance in imaging nAChRs, because administration of radiolabeled nicotine (used for previous imaging studies) resulted in high nonspecific binding and short drug–receptor interaction times (Sihver et al. 2000). 2-[^{18}F]F-A-85380 or simply 2-FA and related compounds (Chefer et al. 1999; Horti et al. 1998; Koren et al. 1998) are being used for PET imaging, and 5-[$^{123/125}$I]iodo-A85380 is being used for SPECT imaging (Chefer et al. 1998; Horti et al. 1999; Mukhin et al. 2000) of $\alpha_4\beta_2$nAChRs.

Studies of nonhuman primates and humans have examined distributions of nAChRs with these new radiotracers, and found regional densities of these receptors similar to those in the animal work cited above (Chefer et al. 2003, 1999; Fujita et al. 2002, 2003; Kimes et al. 2003; Valette et al. 1999). Two recent studies on baboons examined effects of nicotine or tobacco smoke on nAChR availability. In a 2-FA PET study (Valette et al. 2003), IV nicotine (0.6 mg), inhalation of tobacco smoke from one cigarette (0.9 mg nicotine), and IV nornicotine were all found to reduce the volume of distribution of the tracer by roughly 30–60% in the thalamus and putamen at 80 min, and this reduction of 2-FA binding was relatively long lived (up to 6 h). Similarly, a 50% reduction in nAChR availability was found with IV nicotine administration to baboons using an epibatidine analog and PET scanning (Ding et al. 2000). Taken together, these studies demonstrate that radiotracers for nAChRs can be administered safely to measure nAChR densities, and that nicotine and smoking substantially decrease $\alpha_4\beta_2$nAChR availability.

In a recent study (Brody et al. 2006), human cigarette smokers were studied using 2-FA and PET scanning. In this study, only one to two puffs of a cigarette resulted in

50% occupancy of brain $\alpha_4\beta_2$nAChRs, and this occupancy lasted for at least 3.1 h after smoking. Smoking a full cigarette resulted in 88% occupancy, and was accompanied by a reduction in cigarette craving. Binding of nicotine to $\alpha_4\beta_2$nAChR causes desensitization of these receptors, and this 2-FA PET study indicated that smoking may lead to withdrawal alleviation by maintaining nAChRs in the desensitized state.

[^{123} I]5-IA or simply 5-I-A is a SPECT radioligand that binds to β_2nAChRs. In a recent study, Staley et al. (2006) hypothesized that an abnormally high number of β_2nAChRs in early abstinence may be responsible for continued tobacco usage. In this study, 16 smokers and 16 nonsmokers underwent 5-I-A SPECT scanning. Smokers were imaged in the abstinent phase, 7 days after their last cigarette. Each group consisted of seven men and nine women who were matched for age. Women smokers and nonsmokers were also matched by phase of menstrual cycle. Smokers quit cigarettes with brief behavioral counseling, and no medication was used for smoking cessation. In this study, recently abstinent smokers were found to have significantly higher 5-I-A uptake in the striatum, parietal cortex, frontal cortex, anterior cingulate, temporal cortex, occipital cortex, and cerebellum, which suggests that smoking upregulates the number of β_2nAChRs.

2.5 Glutamatergic (and Other) Effects of Nicotine/Cigarette Smoking

Recent autoradiography studies of rodents have examined the effects of nicotine/smoking in other neurotransmitter systems that may be activated by nAChR stimulation. For example, in response to nicotine, glutamate release has been demonstrated in the prelimbic prefrontal cortex (Gioanni et al. 1999), and glutamate and aspartate release have been demonstrated in the VTA (Schilstrom et al. 2000). The finding of nAChR-induced glutamate release in the prefrontal cortex has also been demonstrated by measuring spontaneous excitatory postsynaptic currents (Lambe et al. 2003). Importantly, one of these studies (Gioanni et al. 1999) also demonstrated that nicotine administration facilitates thalamo-cortical neurotransmission through stimulation of nAChRs on glutamatergic neurons.

3 Brain Function Responses to Chronic Nicotine Administration and Cigarette Smoking

3.1 Functional Brain Imaging of Cigarette Craving

As for brain imaging studies of chronic tobacco/nicotine dependence, cigarette smokers experience craving for cigarettes (urge to smoke) within minutes after the last cigarette, and the intensity of craving rises over the next 3–6 h (Jarvik et al.

2000; Schuh and Stitzer 1995). Cigarette-related cues have been shown to reliably enhance craving during this period, compared to neutral cues (Carter and Tiffany 1999).

Two studies used a cigarette versus neutral cue paradigm paired with functional imaging to evaluate brain mediation of cigarette craving. In one study (Due et al. 2002), six smokers and six nonsmokers underwent event-related fMRI when presented with smoking-related images (color photographs) compared with neutral images, for 4 s each. For the smoker group, craving increased during the testing session and exposure to smoking-related images resulted in activation of mesolimbic (right posterior amygdala, posterior hippocampus, VTA, and medial thalamus) and visuospatial cortical attention (bilateral prefrontal and parietal cortex and right fusiform gyrus) circuitry, whereas the nonsmoker group did not have these changes. In the second study (Brody et al. 2002), 20 smokers and 20 nonsmokers underwent two FDG–PET sessions. For one PET session, subjects held a cigarette and watched a cigarette-related video, while for the other, subjects held a pen and watched a nature video (randomized order) during the 30-min uptake period of FDG. When presented with smoking-related (compared to neutral) cues, smokers had higher regional metabolism in bilateral (ACC), left orbitofrontal cortex (OFC), and left anterior temporal lobe. Change in craving scores was also positively correlated with change in metabolism in the OFC, dorsolateral prefrontal cortex, and anterior insula bilaterally.

Taken together, these studies of cigarette craving indicate that immediate responses to visual smoking-related cues (fMRI study) activate the brain reward system, limbic regions, and the visual processing system, while longer exposure to cues (FDG–PET study) leads to activation of the ACC, which mediates anxiety, alertness, and arousal (Chua et al. 1999; Critchley et al. 2001; Kimbrell et al. 1999; Naito et al. 2000; Rauch et al. 1999) and the OFC, which functions in part as a secondary processing center for sensory information (Rolls et al. 1998; Rolls and Baylis 1994).

In a related preliminary study, 17 smokers underwent the same FDG–PET craving versus neutral cue protocol as in the second study of craving listed above (Brody et al. 2002) after treatment with a standard course of bupropion HCl (tapered up to 150 mg orally twice a day for a mean of 5.6 weeks). This group of treated subjects had a significant reduction in smoking levels from pre- to post-treatment (mean 27.1 down to 3.7 cigarettes per day). These treated smokers also had reduced cigarette cue-induced craving and diminished ACC activation when presented with cigarette-related cues, compared to untreated smokers (Brody et al. 2004a). This diminished ACC activation was due to elevated baseline-normalized ACC activity in treated smokers, giving an indication that bupropion treatment of smokers increases resting ACC metabolism.

A more recent study examined (Brody et al. 2007) brain activation during resistance of the urge to smoke when smokers were presented with cigarette-related cues. In this study, activation was found in the cigarette cue resist condition compared with the cigarette cue crave condition in the left dorsal ACC, posterior cingulate cortex (PCC), and precuneus. Other findings of this study include lower magnetic resonance signal for the cigarette cue resist in the cuneus bilaterally, left lateral occipital

gyrus, and right postcentral gyrus. These activations and deactivations were stronger when the cigarette cue resist condition was compared with the neutral cue condition. The urge to smoke scale (craving) score had positive correlations with MR signal in the medial aspect of superior frontal gyrus, supramarginal gyrus, precuneus, inferior frontal gyrus/anterior insula, bilateral corpus callosum, left precentral gyrus, putamen, and middle frontal gyrus, and right lingual gyrus extending to the fusiform gyrus. Negative correlations were found for the cuneus, left occipital gyrus, anterior temporal lobe, postcentral gyrus, insula, and right angular gyrus. This study concludes that active suppression of craving during cigarette cue exposure is associated with activation of limbic and related brain regions and deactivation of primary sensory and motor cortices.

3.2 Functional Brain Imaging of Cigarette Withdrawal

Abstinence-induced changes have also been studied (McClernon et al. 2005) in 13 dependent smokers using event-related fMRI. FMRI images were taken after usual smoking and following overnight abstinence. Self-reported craving measures were also conducted before, during, and after scanning. Results revealed larger hemodynamic responses to smoking compared to control cues in ventral anterior cingulate gyrus and superior frontal gyrus. Results show that brain responses to smoking cues, while relatively stable at the group level following short-term abstinence, may be modulated by individual differences in craving in response to abstinence, particularly in regions subserving attention and motivation.

Rose et al. (2007) also studied smokers ($n = 15$) with functional brain imaging following treatment for nicotine dependence. In this study, subjects were given nicotine patches and denicotinized cigarettes. PET scans were obtained at baseline, after 2 weeks of nicotine patch and denicotinized cigarettes, and 2 weeks after patients returned back to smoking. Craving of cigarettes was lower at the second session compared to the other two. After 2 weeks' exposure to nicotine patches and denicotinized cigarettes, the authors found decreased brain metabolic activity in the right hemisphere anterior cingulate cortex.

Brain activity changes (measured with fMRI) during cigarette withdrawal were recently reported for nicotine-dependent rats (Shoaib et al. 2004). In this study, subcutaneous mecamylamine (1 mg kg^{-1}), a nicotine receptor antagonist, was administered to precipitate withdrawal during scanning, and this state was compared to a control state after subcutaneous saline administration. After subcutaneous mecamylamine, nicotine-dependent rats had bilateral increases in NAc activity compared to the control state.

3.3 Monoamine Oxidase (MAO) Function in Smokers

Fowler and colleagues have performed a series of important studies demonstrating decreases in MAO A and B activity in cigarette smokers using the PET tracers

[^{11}C]clorgyline (Fowler et al. 1996b) and [^{11}C]L-deprenyl-D2 (Fowler et al. 1996a, 1998b), respectively. When compared to former smokers and nonsmokers, average reductions for current smokers are 30 and 40% for MAO A and B (Fowler et al. 2003a). These reductions were the result of chronic smoking behavior rather than a single administration of intravenous nicotine (Fowler et al. 1998a) or smoking a single cigarette (Fowler et al. 1999, 2000, 2005), and are less than those seen with antidepressant MAO inhibitors (Fowler et al. 1994, 1996b). MAO A levels were found to be reduced up to 50% in peripheral organs (heart, lungs, and kidneys) in smokers when compared to nonsmokers. Additionally, a human postmortem study of chronic smokers demonstrated a modest reduction in MAO A binding that did not reach statistical significance (Klimek et al. 2001). Peripheral MAO B is also reduced in cigarette smokers (Fowler et al. 2003b).

MAO participates in the catabolism of dopamine, norepinephrine, and serotonin (Berlin and Anthenelli 2001; Fowler et al. 2003a), and it has been postulated that some of the clinical effects of smoking are due to MAO inhibition, leading to decreases in monoamine breakdown with a subsequent increase in monoamine availability (Berlin and Anthenelli 2001). Thus, smoking may enhance DA availability and the rewarding properties of smoking both through DA release (as described above) and MAO inhibition. Smoking may also alter mood and anxiety through MAO inhibition effects on norepinephrine and serotonin availability and turnover. Comprehensive reviews of the role of MAO in tobacco dependence have recently been published (Berlin and Anthenelli 2001; Fowler et al. 2003a).

4 Discussion: Functional Neuroanatomy of Tobacco Use and Dependence

Both acute and chronic effects of nicotine/cigarette exposure have been elucidated with functional brain imaging. Replicated responses to acute administration of nicotine/smoking include a reduction in global brain activity (perhaps most prominently in smokers with high levels of hostility as a personality trait); activation of the prefrontal cortex, thalamus, and visual system; activation of the thalamus and visual cortex (and possibly ACC) during visual cognitive tasks; and increased DA concentration in the ventral striatum/NAc. Replicated responses to chronic nicotine/cigarette exposure include decreased MAO A and B activity and a substantial reduction in $\alpha_4\beta_2$ nAChR availability in the thalamus and putamen (accompanied by an overall upregulation of these receptors).

This group of findings demonstrates a number of ways in which smoking might enhance neurotransmission through cortico–basal ganglia–thalamic circuits (Alexander et al. 1990), in addition to demonstrating direct effects of chronic nicotine exposure on nAChR availability (Fig. 1). Given that the thalamus (Groenewegen et al. 1999; Herrero et al. 2002; Sommer 2003) and ventral striatum/NAc (Groenewegen et al. 1999; Herrero et al. 2002) function as relay

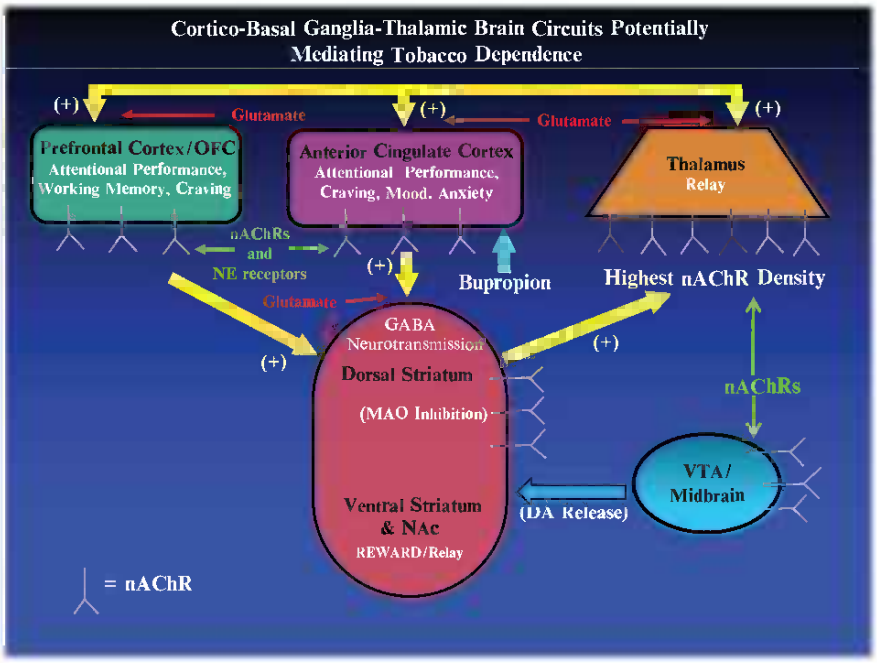

Fig. 1 Representation of the cortico–basal ganglia–thalamic brain circuitry that may mediate the effects of nicotine/smoking on attentional control, craving, mood, and anxiety. Potential targets for nicotine/smoking to enhance attention (and improve craving, mood, and anxiety) include (1) direct stimulation of nicotinic acetylcholine receptors (nAChRs) in cortex, (2) stimulation of the nAChR-rich thalamus and basal ganglia, (3) activation of dopaminergic mesolimbic reward pathways originating in the VTA and projecting to the striatum, and (4) monoamine oxidase (MAO) inhibition in the basal ganglia. *NAc* nucleus accumbens; *VTA* ventral tegmental area

centers for information and for paralimbic and motor processing in the brain, the net effect of smoking may be to enhance neurotransmission along cortico–basal ganglia–thalamic loops originating in the paralimbic cortex. Neurotransmission through these circuits may be stimulated directly by the interconnected (Sherman 2001; Sillito and Jones 2002) nAChR-rich thalamus and visual systems, and/or indirectly through effects on MAO inhibition and DA release in the ventral striatum/NAc, as well as through nicotine stimulation of excitatory glutamatergic input to the dopaminergic system (Mansvelder et al. 2002). In the thalamus, for example, nicotine has direct agonist action on excitatory thalamocortical projection neurons and local circuit neurons, although nicotine also stimulates GABAergic interneurons, so that the relationship between nicotine stimulation and thalamocortical stimulation may be complex (Clarke 2004). There is mixed evidence as to whether or not nicotine stimulates corticothalamic neurons (Clarke 2004).

Enhancement of neurotransmission through prefrontal and paralimbic cortico–basal ganglia–thalamic circuits may account for the most commonly reported

cognitive effect of cigarette smoking, namely, improved attentional performance (Newhouse et al. 2004), and also related effects, such as improvements in reaction times (Hatsukami et al. 1989; Pritchard et al. 1992; Shiffman et al. 1995), arousal (Parrott and Kaye 1999), motivation (Powell et al. 2002), and sustained attention (Rusted et al. 2000). Prefrontal (including both dorsolateral and ventrolateral) (Duncan and Owen 2000; Rees and Lavie 2001; Smith and Jonides 1999) and ACC (Carter et al. 1999; Duncan and Owen 2000; Peterson et al. 1999; Smith and Jonides 1999) cortices are reported to activate during attentional control tasks (especially visuospatial tasks) (Pessoa et al. 2003). Cigarette smoking may enhance attentional control through direct stimulation of nAChRs within these structures or perhaps through subcortical stimulation of nAChRs in the thalamus and via DA release and/or MAO inhibition in the basal ganglia.

In addition to improvement in attention, smoking improves withdrawal symptoms, such as depressed mood, anxiety, and irritability in tobacco-dependent smokers (Cohen et al. 1991; Parrott 2003), and all these effects depend (at least in part) on the expectations of the smoker (Perkins et al. 2003). Though nicotine administration generally results in increased activity along prefrontal and paralimbic brain circuits, it is interesting that both increased and decreased ACC activation during cognitive task performance has been reported (see Sect. 2.2). ACC activity has been associated with anxiety and mood, with increased activity being associated with greater anxiety (Chua et al. 1999; Kimbrell et al. 1999) and decreased activity being associated with depressed mood (Drevets et al. 1997). This combination of findings suggests a potential interaction between expectation of the effects of smoking (e.g., mood improvement, anxiety reduction, or decreased irritability) and direction of ACC activity change during cognitively demanding tasks. Perhaps smokers who expect to and do have anxiety alleviation from smoking have deactivation or decreased activation of the ACC while performing cognitive tasks, whereas those who expect to and do experience mood improvement from smoking have increased activation of the ACC.

In addition to these primary effects of nicotine and smoking, other functional imaging studies reviewed here focus on smoking-related states, such as cue-induced cigarette craving. Such studies are part of a large body of literature examining cue-induced craving for addictive drugs. Studies specific for cigarette cues/craving reveal that exposure to visual cigarette cues immediately activates mesolimbic (VTA, amygdala, and hippocampus) and visuospatial cortical attention areas of the brain, and acutely (over a 30-min period) activate paralimbic regions (ACC and OFC), and that this cue-induced activation may be diminished by a course of bupropion treatment. These results are similar to those of functional imaging studies for drugs other than tobacco (Goldstein and Volkow 2002; Miller and Goldsmith 2001), and it has been posited that at least some of the activations seen with cigarette-related cues (cortical attention areas and OFC) are associated with an expectation of smoking in the nontreatment-seeking subjects who participated in these studies (Wilson et al. 2004).

5 Future Directions

New radioligands are in development for nAChRs. Currently, 2-FA, 6-FA, and 5-I-A radiotracers are available, which have affinity to bind to the $\alpha_4\beta_2$ nAChR subtype. Other radiotracers are in development for this subtype, but there is need for radioligands for imaging of other subtypes of nicotinic receptors, including the α_7 subtype, which is abundant in humans. Future research is likely to focus on radioligands for imaging $\alpha_4\beta_2$ nAChR in the thalamus with faster kinetics than 2-FA, 6-FA, and 5-I-A. Radiolabeled antagonists for imaging of $\alpha_4\beta_2$ nAChR may prove very beneficial for greater understanding of receptor binding and ultimately in development of pharmacological agents to help with quitting smoking (Pomper et al. 2005; Horti et al. 2006).

New treatments are being discovered for smoking cessation, and the Food and Drug Administration has recently approved varenicline, which is a partial nAChR agonist and antagonist. The agonist effect is caused by binding to nicotinic receptors and stimulating receptor-mediated activity. The antagonist effect occurs when varenicline blocks the ability of nicotine to activate nicotinic receptors. Imaging studies with varenicline may tell us more about nicotine dependence and the role of the $\alpha_4\beta_2$ nicotine receptor.

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Molecular and Cellular Mechanisms of Action of Nicotine in the CNS

Jacques Barik and Susan Wonnacott

Contents

Abstract Nicotine achieves its psychopharmacological effects by interacting with nicotinic acetylcholine receptors (nAChRs) in the brain. There are numerous subtypes of nAChR that differ in their properties, including their sensitivity to nicotine, permeability to calcium and propensity to desensitise. The nAChRs are differentially localised to different brain regions and are found on presynaptic terminals as well as in somatodendritic regions of neurones. Through their permeability to cations, these ion channel proteins can influence both neuronal excitability and cell signalling mechanisms, and these various responses can contribute to the development or maintenance of dependence. However, many questions and uncertainties remain in our understanding of these events and their relevance to tobacco addiction. In this chapter, we briefly overview the fundamental characteristics of nAChRs that are germane to nicotine's effects and then consider the cellular responses to acute and chronic nicotine, with particular emphasis on dopamine systems because they have been the most widely studied in the context of nicotine dependence. Where appropriate, methodological aspects are critically reviewed.

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1 Introduction

Inhaled nicotine is efficiently delivered to the brain (see chapter by Benowitz, this volume) where it selectively interacts with its central targets, the neuronal nicotinic acetylcholine receptors (nAChRs). The multiple subtypes of nAChR (see chapter by Collins et al., this volume) all bind nicotine but with different affinities, depending on the subunit composition of the nAChR. Binding may result in activation or desensitisation of nAChRs, reflecting the temporal characteristics of nicotine delivery and local concentration of nicotine. Another level of complexity of the actions of nicotine reflects the widespread and non-uniform distribution of nAChR subtypes within the brain, such that nicotine can influence many centrally regulated functions in addition to the reward systems. In this chapter, we address the consequences of nicotine interactions with nAChRs at the molecular, cellular and anatomical levels. We critically evaluate experimental approaches, with respect to their relevance to human smoking, and contrast the acute and chronic effects of nicotine.

2 Acute Effects of Nicotine on nAChRs

2.1 Molecular Interactions

2.1.1 Nicotine Binding to nAChRs

nAChRs are ligand-gated cation channels (Fig. 1). Binding of the neurotransmitter acetylcholine (ACh) or exogenous agonists like nicotine is transduced into an intracellular signal by opening the intrinsic ion channel of the nAChR, allowing the flow of cations through the receptor. An nAChR can exist in multiple, discrete, interconvertible, conformational states; the kinetics of the transitions between states range from milliseconds to minutes, and can differ substantially between nAChR subtypes (see below). Amongst all the putative allosteric transitions, nAChRs oscillate between four dominant states: the resting state (R: channel closed and agonist binding site unoccupied), the active state (A: channel open), the desensitised state (D: channel closed and agonist bound with high affinity) and the inactive state (I: a long-lasting desensitised state) (Changeux et al. 1998; Fig. 1a). Under brief exposure to relatively high concentrations of ACh or nicotine, the equilibrium shifts towards the "A" state, allowing signal transduction, before the nAChR desensitises. However, under prolonged exposure to agonist (e.g. the relatively stable plasma nicotine concentrations sustained by smokers during the smoking day, or nicotine delivered by various NRT products), or application of low agonist concentrations, the desensitised states are more likely to be stabilised, making nAChRs refractory to activation and preventing receptor signalling.

Early studies of muscle nAChRs established that the receptor has two agonist binding sites and both must be occupied for efficient channel opening to occur.

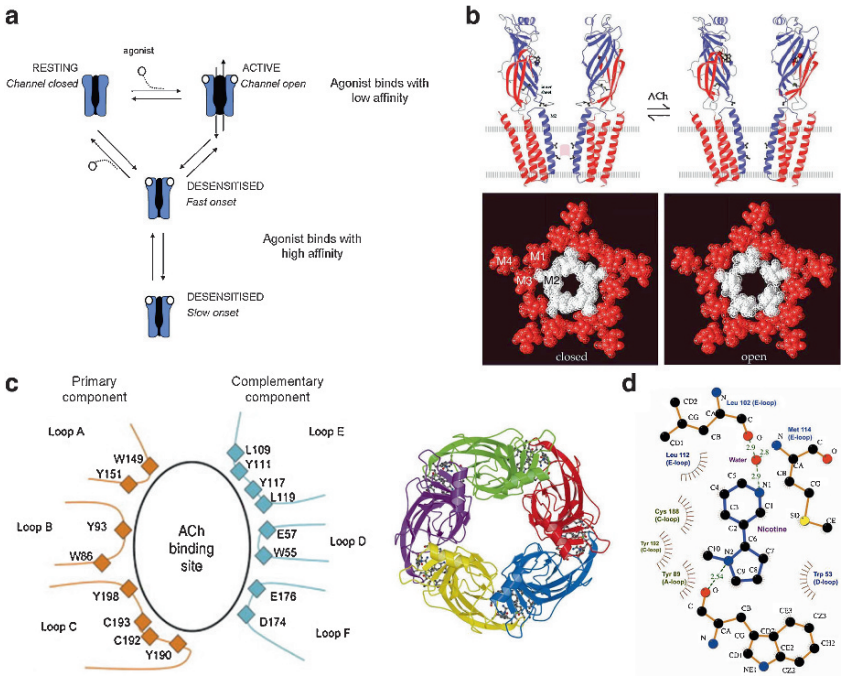

Fig. 1 Molecular characterization of the nAChR. **a** Multiple states of the nAChR. In the absence of agonist, the nAChR is predominantly in the closed state, with the integral ion channel closed. This is the "resting" state that is responsive to the application of agonist. Binding of agonist stabilises the channel in the open configuration, allowing cation flux across the membrane. The open state can be short-lived (depending on nAChR subtype and agonist concentration), such that the nAChR undergoes a further series of transitions to a desensitised state in which agonist remains bound (with high affinity) while the channel is closed. In the desensitised state, the nAChR is refractory to activation. The removal of agonist is accompanied by a return to the resting closed state. **b** Structural models of the nAChR, derived from high-resolution electron microscopy of *Torpedo* nAChR. *Upper panel*: two α subunits are depicted; the other three subunits are omitted for clarity. The protein traverses the membrane by adopting an α-helical conformation to create a central pore or channel (the M2 transmembrane spanning segments that line the channel are coloured *blue*). The extracellular portion of the nAChR that contains the ligand binding domain is predominantly β sheet conformation. The binding of ACh results in structural movements that create a rotational effect, resulting in widening of the channel at the region of the hydrophobic "gate". This is illustrated in the *lower panel*, which shows a cross-section of the channel at the level of the gate, viewed from the extracellular side. In the open state, the channel is widened by approximately 3 Å. From Unwin (2003), with permission from Elsevier. **c** The agonist binding site. *Left panel*: schematic illustrating the localisation of the ACh binding site at the interface of an α subunit (that provides the "primary" component) and the adjacent subunit (the "complementary" component). Each subunit contributes conserved amino acids, identified by biochemical and mutagenesis experiments, located in three non-contiguous loops in each subunit. Modified from Changeux and Taly (2008), with permission from Elsevier. *Right panel*: crystal structure of the AChBP, viewed from above, with each subunit represented in a different colour. The localisation of the binding site residues of loops A–F is indicated in ball-and-stick representation and confirms that they lie at the interface between adjacent subunits to create a binding pocket accessible from the exterior surface of the protein. From Brejc et al. (2001), with permission from Macmillan Publishers. **d** Nicotine docked in a binding site of the AChBP. The AChBP was co-crystallised with nicotine bound. Interactions with some of the key amino acids from loops A–F that are important for nicotine binding are indicated (AChBP numbering differs slightly from nAChR numbering of these residues in c.). The pyridine nitrogen of nicotine forms a hydrogen bond with a water molecule. From Celie et al. (2004), with permission from Elsevier

The binding sites were correlated with the two α subunits in muscle nAChRs. Numerous subsequent studies, notably those involving mutagenesis and/or affinity labelling experiments, have demonstrated that the agonist-binding site resides at the interface between the α subunit and an adjacent subunit. The α subunit contributes the "primary component" of agonist binding, comprising three non-contiguous polypeptide loops (named A, B, C) located in the N-terminal region. The N-terminal domain of the adjacent subunit provides the "complementary component" (D, E, F loops; Fig. 1c) (Blount and Merlie 1989; Corringer et al. 2000; Karlin 2002). This model has been corroborated by X-ray crystallography of a molluscan glial-derived homopentameric ACh-binding protein (AChBP; Fig. 1c) (Brejc et al. 2001; Smit et al. 2001). The AChBP has homology with the N-terminal extracellular domain of nAChRs, most closely resembling that of the homopentameric α7 nAChR. The key residues of loops A–F are highly conserved, creating a binding site that is remarkably similar to that of nAChRs. Co-crystallisation of the AChBP with nicotine has revealed that binding involves conformational changes in the ligand binding site, with the largest movement in the C loop (Celie et al. 2004; Fig. 1d). The higher affinity for nicotine (compared with carbamoylcholine) reflects the ability of the pyrrolidine and pyridine nitrogens to form hydrogen bonds with the B loop and a water molecule, respectively, together with more hydrophobic interactions. The first atomic level (1.94 Å) view of a (partial) nAChR binding site has recently come from the crystallisation of the extracellular domain of a muscle α1 nAChR subunit complexed with α-bungarotoxin (αBgt) (Dellisanti et al. 2007).

Heteromeric neuronal nAChRs are composed of α and β subunits. The α subunits are distinguished by the presence of adjacent (vicinal) cysteine residues in loop C (Fig. 1c), and this originally defined α subunits as agonist-binding subunits (Corringer et al. 2000; Gotti et al. 2006). As both α and the adjacent non-α subunit determine the agonist binding site, different β subunits can contribute to the pharmacological differences between neuronal nAChR subtypes. For example, expression of pairwise combinations of α and β subunits resulted in striking differences in the potency and efficacy of nicotine between β2- and β4-containing nAChRs (Luetje and Patrick 1991). As well as such differences between nAChRs, the agonist binding sites within an nAChR can differ. This has been clearly documented for muscle nAChR (in which the two α subunits are partnered by γ/ε or δ subunits): the αδ binding site has markedly higher affinity for agonists and lower affinity for antagonists than the αγ site (Blount and Merlie 1989; Karlin 2002). It is likely that similar non-equivalence of binding sites occurs in neuronal heteromeric nAChRs: among the various nAChRs expressed in rodent dopamine neurones (see chapter by Collins et al., this volume), α4β2 nAChRs will have two identical agonist-binding sites, whereas α4α6β2β3 nAChRs will have two different binding sites (α4β2 and α6β2 interfaces). The latter subtype has higher sensitivity to nicotine than α4β2 nAChRs (EC_{50} 0.2 μM) (Salminen et al. 2007).

The homomeric α7 nAChR presents a special case, as each subunit contains both primary and complementary components of the binding site, with the possibility of five agonist-binding sites per receptor (Palma et al. 1996). It is generally regarded as having lower sensitivity to agonist and desensitising rapidly. However, recent studies

(in which mutated binding sites could be sequentially inactivated by a sulfydryl reagent) suggest that having five binding sites gives the α7 nAChR a greater range of sensitivity: low concentrations of agonist sufficient to occupy as few as one or two binding sites can effectively activate the α7 nAChR, while higher concentrations that occupy more binding sites promote rapid, albeit short-lived, desensitisation (Papke et al. 2007).

2.1.2 Signal Transduction: Channel Opening

While atomic resolution crystal structures provide detailed descriptions of the nAChR, they represent only the most stable states (resting or desensitised) due to the time required for crystallisation to occur. The pioneering work of Nigel Unwin, using electron microscopy to visualise intact nAChRs that form semi-crystalline arrays in *Torpedo* electroplax, has afforded an opportunity to capture the open state of the nAChR, albeit at the lower resolution of 4.6 Å (Fig. 1b). This was achieved by spraying ACh onto the receptor at the moment of rapid freezing (Unwin 1995, 2003). Comparison with the closed state (the resting or desensitised/inactive state) of the nAChR has provided insight into the changes induced by agonist binding that lead to channel opening.

In the closed or non-conducting state, the lumen of the channel is blocked by a molecular barrier or "gate", preventing the flux of cations. The pore is lined by the second transmembrane domain, M2, of each of the five subunits that make up the nAChR. Unwin and colleagues located the resting gate in the mid-region of M2, where the lumen narrows and is only 6 Å wide, too narrow for hydrated cations to pass through and energetically unfavourable for the removal of hydration shells (Unwin 1995; Miyazawa et al. 2003). In this model, the gate consists of a ring of leucine residues and a ring of valine or isoleucine residues one helix-turn above. The binding of agonist destabilises the hydrophobic intersubunit interactions, allowing the α subunit N-terminal domains to rotate by 15°. As a consequence, the five M2 α-helices change orientation, widening the channel by 3 Å, sufficient for monovalent and divalent cations (K^+, Na^+, Ca^{2+}) to diffuse through the water-filled pore (see Fig. 1b). Application of the substituted-cysteine accessibility method (SCAM) has predicted that the gate is lower down the channel than Unwin's proposed leucine ring (Wilson and Karlin 1998; Karlin 2002). Moreover, the biochemical approach distinguished separate resting and desensitised gates, consistent with electrophysiological data (Auerbach and Akk 1998). Although the physical location of the gate (or gates) requires confirmation, the principles of channel opening derived from the electron microscopy approach are widely accepted, leading to the "quaternary twist" model of channel opening (Changeux and Taly 2008).

The 1.6 Å-resolution crystal structure of the muscle α1 subunit extracellular domain revealed an unexpected water molecule in the core of the α1 nAChR subunit, in the vicinity of the transmembrane domain (Dellisanti et al. 2007). This is surrounded by hydrophilic residues that are conserved in nAChR subunits, but absent from the AChBP. The water molecule confers flexibility, making the "non-optimally

packed core" of the nAChR predisposed to undergo conformational change on agonist binding, a response that is not required by the AChBP that lacks an ion channel. Site-directed mutational analysis has implicated the amino acids around this hydrophilic cavity in the gating process. In addition, sugars arising from the conserved N-linked glycosylation of nAChR subunits are also important for the coupling of agonist binding to channel gating (Dellisanti et al. 2007). Glycans may modulate the duration of channel open and closed states; different glycosylation patterns between nAChR subtypes could provide another source of functional heterogeneity. The highly variable cytoplasmic loop between transmembrane domains 3 and 4 also has the potential to differentially influence the gating characteristics of different nAChR subtypes (Peters et al. 2006).

Although nAChR subunits share common structural features and conserved amino acids that are involved in ligand binding, channel opening and conduction, the unique amino acid sequence of each subunit will influence nAChR function (Dani and Bertrand 2007). In addition, the absence of key amino acids will prevent certain subunits from contributing to the binding pocket. This appears to be the case for the α5 nAChR subunit, in which an essential tyrosine residue (corresponding to Tyr 190, Fig. 1c) is absent from the C-loop, being replaced by an aspartate residue that cannot contribute to the primary binding component. Similarly, β3 subunits, in which the tyrosine residues of the complementary binding site loop E (Fig. 1c) are replaced by phenylalanine, do not participate in agonist binding (Corringer et al. 2000): α5 and β3 subunits are believed to provide the fifth subunit in a pentamer, the role of the β1 subunit in muscle nAChRs. However, incorporation of these "accessory" subunits can significantly modulate the properties of heteromeric nAChRs (Kuryatov et al. 2008). For example, inclusion of an α5 subunit enhances the Ca^{2+} permeability of α3β2 or α3β4 nAChRs (Gerzanich et al. 1998), decreases the EC_{50} for nicotine of α4β2 nAChRs (Ramirez-Latorre et al. 1996), and affects upregulation in response to nicotine (see Sect. 3.4.3). Indeed, the stoichiometry of α4β2 nAChRs without any additional subunits (i.e., with either α4 or β2 as the accessory subunit) affects agonist affinity (see Sect. 3.4.2).

2.2 Cellular Consequences of Activating nAChRs

2.2.1 Changes in Intracellular Na^+ and Ca^{2+}

Activation of nAChRs results in the influx of Na^+ and Ca^{2+}, which engenders rapid changes in membrane potential and local increases in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. It is the ability to alter $[Ca^{2+}]_i$ that gives nAChRs a pivotal role in modulating cellular functions (Berridge et al. 2000; Dajas-Bailador and Wonnacott 2004). The subunit composition of the nAChR dictates its intrinsic Ca^{2+} permeability, expressed as "fractional Ca^{2+} current" (P_f), an experimental estimate based on synchronised recordings of membrane potential and fluorescent signals gathered using Ca^{2+}-sensitive dyes (Fucile 2004). Heteromeric neuronal nAChRs

(excluding α9/α10 nAChRs) are moderately permeable to Ca^{2+} with P_f values of 1.5–5%, whereas for homomeric α7 nAChRs the positioning of charged and polar amino acids at the cytoplasmic end of M2 confers a Ca^{2+} permeability comparable to that of heteromeric NMDA receptors (P_f∼6–12%) (Vernino et al. 1992; Bertrand et al. 1993; Seguela et al. 1993).

In addition, nAChRs can further augment $[Ca^{2+}]_i$ by secondary activation of voltage-operated calcium channels (VOCC), or by mobilizing the release of Ca^{2+} from internal stores via a mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) (Tsuneki et al. 2000; Sharma and Vijayaraghavan 2003; Dajas-Bailador and Wonnacott 2004). Several reports support the differential coupling of homomeric and heteromeric nAChRs to distinct Ca^{2+} pathways. Non-α7 nAChRs primarily trigger activation of VOCCs; examples include β2-containing nAChRs on dopamine cell bodies (Tsuneki et al. 2000) and terminals (Soliakov and Wonnacott 1996; Turner 2004; Nayak et al. 2001). In contrast, α7 nAChRs preferentially elicit CICR from ryanodine-sensitive stores in numerous preparations (Dajas-Bailador and Wonnacott 2004; Dickinson et al. 2007, 2008). The ability to provoke CICR is likely to reflect the higher Ca^{2+} flux through α7 nAChRs and may also require a spatial relationship between α7 nAChRs and Ca^{2+} stores to facilitate their communication.

The significance of coupling to either VOCC or CICR is that different nAChRs can generate Ca^{2+} signals with distinct kinetic, temporal and spatial characteristics. Hence, the nicotinic modulation of this ubiquitous intracellular messenger places nAChRs in a key position to influence a variety of Ca^{2+}-dependent neuronal processes, ranging from neurotransmitter release to synaptic plasticity and gene transcription (Berridge et al. 2000; Dajas-Bailador and Wonnacott 2004).

2.2.2 Subcellular Distribution of nAChRs

The consequences of nicotinic signalling are dictated by the subcellular localisation of nAChRs (Fig. 2). It was clear from early studies that a significant proportion of nAChRs in the brain are presynaptic (Wonnacott 1997). Presynaptic nAChRs can directly influence transmitter release. Preterminal nAChRs located at the neck of a synaptic bouton can promote transmitter release by initiating action potentials, and are therefore sensitive to tetrodotoxin (Lena et al. 1993; Dani and Bertrand 2007).

nAChRs on cell soma and dendrites may be postsynaptic to cholinergic nerve terminals but there is limited evidence for nicotinic synaptic transmission in the brain, suggesting that this arrangement is not common (Dani and Bertrand 2007). One example relevant to reward systems is the cholinergic input from the pedunculopontine and laterodorsal tegmental nuclei (see Fig. 3) to midbrain dopamine neurones (Mena-Segovia et al. 2008; Maskos 2008). More commonly, nAChRs appear to be extrasynaptic. In an ultrastructural study, α7 nAChRs have been observed to be perisynaptic around glutamatergic or GABAergic synapses in the CA1 stratum radiatum of the hippocampus (Fabian-Fine et al. 2001). Somatodendritic nAChRs can influence neuronal excitability as a consequence of local depolarisation (excitatory

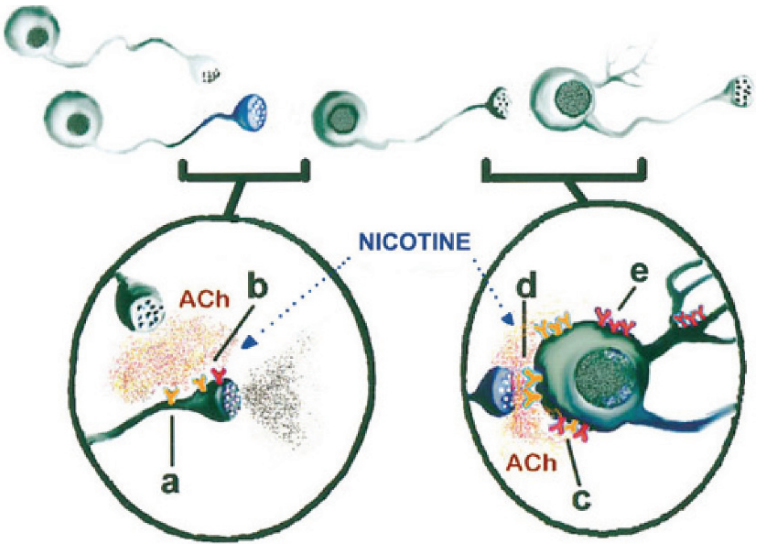

Fig. 2 Subcellular localisation of neuronal nAChRs. *Upper left*: a cholinergic neurone releases ACh in the vicinity of a presynaptic nerve terminal. The *enlarged view* indicates that the nerve terminal might have preterminal **a** or presynaptic **b** nAChRs that can elicit transmitter release when activated. *Upper right*: a cholinergic neurone synapses onto the cell soma of the postsynaptic neurone. The enlarged view indicates that the postsynaptic cell might bear perisynaptic **c**, postsynaptic **d**, or extrasynaptic **e** nAChRs. Dendritic nAChRs are also indicated. Heteromeric nAChRs composed of α and β subunits (*orange*) and homomeric α7 nAChRs (*red*) are distinguished. Nicotine would activate these receptors in the absence of cholinergic activity. Modified from Role and Berg (1996) with permission from Elsevier

postsynaptic potentials) that could result in increased action potential firing, leading to transmitter release in terminal fields. Alternatively, or perhaps it would be more accurate to say additionally, local changes in $[Ca^{2+}]_i$ in response to somatodendritic nAChR activation can influence a variety of cellular processes by altering or promoting Ca^{2+}-dependent signalling events. This includes signalling to the nucleus to effect changes in gene transcription.

The diversity of nAChRs is exemplified in rodent midbrain dopamine neurones (Fig. 3; see Wonnacott et al. 2005). Dopaminergic cell bodies express predominantly heteromeric nAChRs: α4α6α5β2 and α4α5β2 subtypes. In addition, α7 nAChRs are present in fewer than half the neurones (Klink et al. 2001). Dopaminergic terminals in striatum or nucleus accumbens (NAc) possess α4β2, α4α5β2, α4α6β2β3 and α6β2β3 nAChRs (Gotti et al. 2006; see chapter by Collins et al., this volume): it is not clear whether multiple subtypes are present on the same terminal, or whether they are segregated to distinct inputs. The evidence for this diversity comes from the generation of transgenic mice with targeted deletions of specific subunits, in combination with 6-hydroxydopamine lesions of dopaminergic afferents and pharmacological analysis, in addition to single cell RT-PCR, electrophysiological recordings and immunohistochemistry (Jones et al. 2001; Klink et al. 2001; Zoli

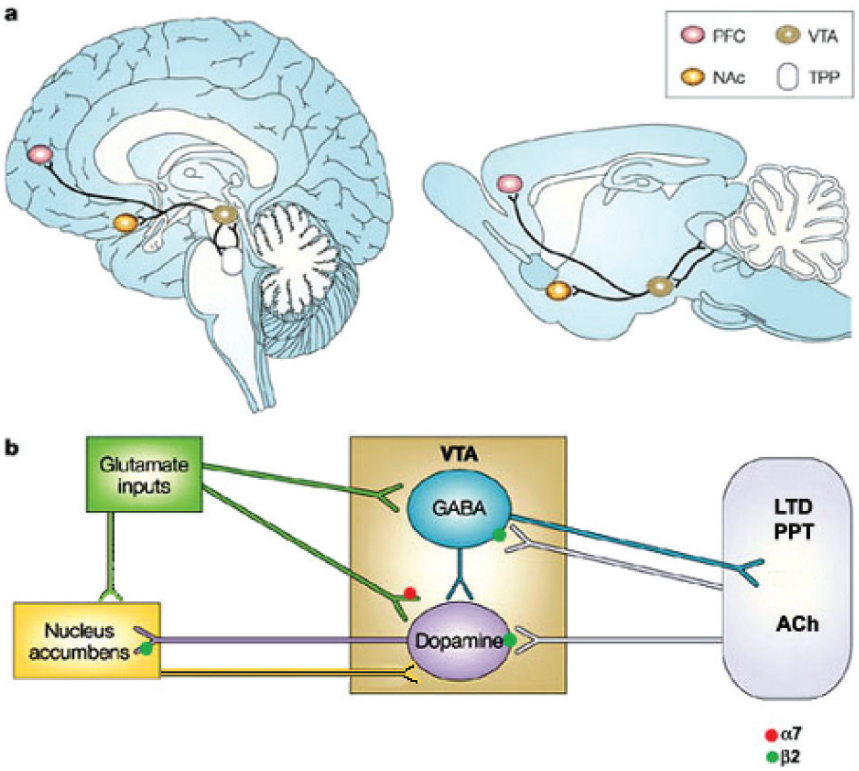

Fig. 3 Localisation of nAChRs in the "reward" pathway. **a** Human (*left*) and rat (*right*) brains showing the dopamine projections from the VTA to the NAc and prefrontal cortex (PFC). Reciprocal connections between the VTA and the tegmental pedunculopontine nucleus (TPP) are also indicated. The TPP comprises the laterodorsal tegmental (*LDT*) and pedunculopontine tegmental (*PPT*) nuclei. **b** Schematic highlighting the major neurotransmitter inputs and outputs of the VTA. The NAc receives predominantly glutamatergic projections from the basolateral nucleus of the amygdala, the hippocampus and thalamus, in addition to cortical inputs (notably from the PFC) (Heimer et al. 1985). Major glutamatergic inputs to the VTA are from the PFC, bed nucleus of the stria terminalis, and the TPP; additional inputs arise from the hypothalamus, ventral pallidum, medial preoptic area and medial septum, as well as some brainstem nuclei (Geisler et al. 2007). The principal known locations of heteromeric β2-containing nAChRs (*green*) and homomeric α7 nAChRs (*red*) within the VTA are indicated. Modified from Laviolette and van der Kooy (2004), with permission from Macmillan Publishers

et al. 2002; Champtiaux and Changeux 2002; Marubio et al. 2003; Cui et al. 2003; Gotti et al. 2005; Salminen et al. 2007). The functional significance of the relatively restricted expression of α6 and β3 subunits to catecholamine neurones and components of the visual system is presently unclear, although the presence of the β3 subunit in presynaptic (but not somatodendritic) α6-containing nAChRs suggests that it may have a targeting role. Indeed, β3-null mutant mice have a reduced expression of α6-containing nAChRs in striatal dopamine terminals (Gotti et al. 2005).

2.2.3 Neurotransmitter Release and Synaptic Plasticity

In vitro and In vivo Methods for Assessing nAChR-evoked Neurotransmitter Release

Several techniques, with intrinsic advantages and disadvantages, have been used to assess the release of neurotransmitters in response to nAChR activation, both in vitro and in vivo. These methods are briefly reviewed here; for a more detailed comparison see Wonnacott et al. (2002).

In vitro methods include electrophysiological recording, superfusion and static release assays. Patch clamp recordings provide the most exquisite sensitivity: chemical, temporal and spatial (Sakmann 2006). However, for presynaptic receptors it is not possible to record directly from presynaptic boutons, due to their small size (except in exceptional cases, such as the calyx of Held; Sakmann 2006). Postsynaptic recordings provide an indirect index of transmitter release, by using the postsynaptic receptors as a detection system. This is most applicable to the nicotinic modulation of glutamate or GABA release, which results in fast postsynaptic potentials. Thus, it is possible to interrogate individual synapses over a millisecond timescale, and this approach has provided much of the evidence for the influence of nAChRs on glutamatergic and GABAergic transmission, notably in the hippocampus and VTA (McKay et al. 2007).

Electrophysiology has proved less useful for characterising the release of other transmitters including the catecholamines, because these do not generate fast postsynaptic currents but signal through G-protein coupled receptors. Electrochemical techniques, such as constant-potential amperometry, high-speed chronoamperometry and fast-scan cyclic voltammetry can monitor local increases in transmitter release with varying degrees of temporal resolution (Michael and Wightman 1999). The carbon fibre microelectrodes employed measure the oxidation/reduction of released neurotransmitter at the electrode surface in response to an applied potential, and this methodology has been mostly used to measure dopamine. The intrinsic properties of the electrodes determine the chemical and temporal resolution of these techniques; fast-scan cyclic voltammetry is capable of subsecond measurements and has been applied to the study of presynaptic nicotinic modulation of dopamine release in the dorsal striatum and NAc (Zhou et al. 2001; Exley et al. 2007). An advantage of this approach is that it permits electrical stimulation of dopaminergic afferents to mimic the intrinsic firing patterns of these neurones, thus providing a more physiological perspective than is possible with other neurochemical in vitro methods.

The superfusion of isolated nerve terminals (“synaptosomes”) is a widely used technique for interrogating the pharmacological properties of presynaptic receptors and their biochemical mechanisms. Physiological buffer continuously flows over a layer of synaptosomes loaded with radiolabelled transmitter, such that released transmitter is removed in the perfusate and collected. A key feature of this methodology is that it eliminates transmitter crosstalk between different boutons, enabling presynaptic events to be studied in isolation (Raiteri and Raiteri 2000).

This methodology has been extensively applied to nAChRs, a reflection of the abundance of presynaptic nAChRs in the brain (Wonnacott 1997), with particular emphasis on striatal dopamine terminals resulting in a thorough characterisation of the various nAChR subtypes associated with nigrostriatal afferents (see chapter by Collins et al., this volume). The temporal resolution of this method is determined by the flow rate of the perfusing buffer and the size of fractions collected, typically 0.5–2 min fractions (Soliakov et al. 1995; Clarke and Reuben 1996; Grady et al. 1997). However, a super-fast technique with subsecond resolution has been described; using this approach, a small α7 nAChR-mediated enhancement of evoked [^{3}H]dopamine release from striatal synaptosomes in response to repeated nicotine applications was reported (Turner 2004). In addition to dopamine release, nicotinic modulation of the release of other transmitters, including noradrenaline (Clarke and Reuben 1996), 5HT (Reuben and Clarke 2000), GABA (Lu et al. 1998), ACh (Wilkie et al. 1996; Grady et al. 2001) and D-aspartate (Marchi et al. 2002; Rousseau et al. 2005; Dickinson et al. 2008), has been documented in synaptosome preparations.

Superfusion of brain slices (or, more correctly, minces or prisms) loaded with radiolabelled transmitter is a robust method; the tissue preparation provides an extra layer of complexity with scope for local interactions between elements within the slice. Comparison with synaptosomes has allowed direct and indirect nicotinic modulation of dopamine release to be dissected (Kaiser and Wonnacott 2000). Superfusion methodology is relatively low throughput, with commercial or custom-built systems having 20 or fewer superfusion chambers operating in parallel. Recently, a high-throughput static release system for the nicotinic modulation of transmitter release from slices was described (Anderson et al. 2000; Puttfarcken et al. 2000). This is carried out in 96-well filter plates equipped with a membrane to support the tissue slices. After exposure to drugs, the bathing medium (containing released transmitter) is removed by vacuum filtration into a 96-well plate (for counting radioactivity) placed beneath the filter plate. In spite of the static nature of the assay, results obtained with this methodology reliably reproduce those obtained by conventional superfusion (Anderson et al. 2000; Barik and Wonnacott 2006). This method has also been employed to monitor [^{3}H]ACh release from synaptosomes (Mogg et al. 2004).

The microdialysis technique (Westerink 2000) has been the most widely used method for monitoring the nicotinic modulation of transmitter release, notably dopamine, in vivo. It combines good anatomical precision (e.g. allowing sampling from either the core or the shell of the NAc; see chapter by Balfour, this volume) with detection of endogenous (not radiolabelled) transmitter in physiological preparations (conscious, freely moving animals). However, it suffers from poor temporal resolution as 10–20 min fractions are needed to provide detectable amounts of transmitter. As well as systemic administration, drugs can be delivered via a cannula into other brain regions, such as cell body areas (Nisell et al. 1994), or locally via the probe by reverse dialysis (Marshall et al. 1997). The latter route allows one to target the terminal field rather than cell bodies. The concentration of drugs reaching nAChRs can only be estimated, and administration via a cannula or dialysis probe will result in a concentration gradient through the surrounding tissue.

Nicotinic Modulation of Neurotransmitter Release

From relatively early studies, catecholaminergic neurones appeared to be important for intracranial self-administration, and midbrain dopamine systems assumed a central role in the reward circuitry purported to underlie drug addiction (Koob 1992). Hence, the release of dopamine (with emphasis on the mesolimbic dopamine system in in vivo studies; see chapter by Balfour, this volume) has received particular attention. The modulation of dopaminergic neurones by glutamatergic afferents and inhibitory GABAergic interneurones or projection neurones (Fig. 3) has stimulated examination of the nicotinic modulation of these transmitters with respect to models of dependence. Nicotine has also been reported to evoke the release of many other neurotransmitters, with varying degrees of relevance to dependence per se (Vizi and Lendvai 1999). The complex effects on neural network function that can ensue from a superficially simple facilitation of transmitter release is exemplified by a recent electrophysiological analysis of nicotine's actions in the prefrontal cortex: the nicotinic enhancement of GABA release from interneurones serves to raise the threshold for synaptic plasticity, with implications for prefrontal information processing and storage (Couey et al. 2007).

Here we will focus on midbrain dopamine systems. In addition to the direct modulation of dopamine release by presynaptic nAChRs (reviewed in chapter by Collins et al., this volume), studies using striatal tissue prisms have provided functional evidence for indirect modulation of dopamine release via spillover of glutamate. In this model, $\alpha 7$ nAChRs on neighbouring cortico-striatal glutamatergic afferents promote the release of glutamate that, in turn, stimulates heterosynaptic ionotropic glutamate receptors on dopaminergic terminals to enhance dopamine release (Kaiser and Wonnacott 2000; Barik and Wonnacott 2006). This model is supported by the demonstration that $\alpha 7$ nAChRs increase the depolarisation-induced release of [^{3}H]D-aspartate, a surrogate for glutamate, from rat and human striatal synaptosomes (Marchi et al. 2002).

The physiological significance of this ability of nicotine to locally enhance dopamine release is not fully appreciated. Systemically administered nicotine elicits dopamine release, but this response is antagonised by mecamylamine delivered to the cell body region (VTA) rather than the terminal field (NAc) (Nisell et al. 1994). Local delivery of nicotine to the NAc or dorsal striatum via the microdialysis probe results in increased dopamine overflow (Nisell et al. 1994; Marshall et al. 1997), so presynaptic nAChRs are capable of influencing dopamine release in vivo. A rationalisation of these apparent contradictions is emerging from recent real-time measurements of dopamine release from striatal slices using fast-scan voltammetry (Zhou et al. 2001; Exley and Cragg 2008). Under different stimulation conditions, to mimic tonic or phasic firing patterns of these neurones, and taking into account the contribution of ACh released from local interneurones, nicotine is proposed to desensitise $\beta 2$-containing nAChRs on dopamine terminals during tonic dopamine release. By removing short-term depression this facilitates greater dopamine release in response to burst firing, thus enhancing the contrast between resting (tonic) activity and stimulated phasic (bursting) activity. The $\alpha 6$-containing nAChRs dominate

these effects of nicotine in the NAc, but have a lesser role, compared to other β2-containing nAChRs, in dorsal striatum (Exley et al. 2007).

The balance between desensitisation and activation of nAChRs is also critical for the actions of nicotine in the VTA. Dani and colleagues demonstrated that the various subtypes of nAChR in the VTA are differentially activated and desensitised by concentrations of nicotine similar to those found in the blood of smokers (Pidoplichko et al. 1997; McKay et al. 2007). In this model, the α4β2 nAChRs that reside on GABAergic interneurones (Klink et al. 2001) desensitise in response to nicotine more readily than the more complex heteromeric nAChRs on the dopaminergic cell bodies (Fig. 3). This effectively reduces GABA release and relieves this inhibitory influence (Mansvelder et al. 2003). Together with activation of α7 nAChRs on glutamatergic afferents to increase glutamatergic activation, the dopamine neurone switches from phasic to burst firing, with greatly increased dopamine release in the NAc (Schilström et al. 2003; Goto et al. 2007). Support for the role of somatodendritic β2-containing nAChRs in this switch to burst firing comes from the observation that it is greatly diminished in knockout mice lacking the β2 nAChR subunit, and is restored by targeted expression of the β2 subunit in the VTA (Mameli-Engvall et al. 2006). As these heteromeric nAChRs receive cholinergic inputs from the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) (Mena-Segovia et al. 2008), this has led to the proposition that cholinergic nicotinic transmission acts as a "gate" that permits this switch to burst firing driven by glutamatergic stimulation (Maskos 2008). In addition to having a key role in nicotine dependence (when nicotine supplants ACh, perhaps accentuating the gate effects by the balance of activation and desensitisation alluded to above), this cholinergic nicotinic gate may contribute to goal-directed behaviours in general, including individual differences in drug abuse liability (Fagen et al. 2007) and cocaine-induced dopamine release (Zanetti et al. 2007).

Presynaptic Plasticity

The activation of somatodendritic β2-containing nAChRs is transient and the receptors desensitise, even at low concentrations of nicotine (Fig. 1a). However, systemic administration of nicotine as a single injection results in long-lasting dopamine release in the NAc (see chapter by Balfour, this volume). Nicotine-induced plasticity at glutamatergic and GABAergic synapses onto mesolimbic dopamine neurones (Fig. 3) has been proposed as a mechanism that translates a brief nicotinic activation into a long-lasting response (Mansvelder et al. 2003; McKay et al. 2007).

Functional and ultrastructural studies support the presence of α7 nAChRs on glutamatergic afferents to the VTA, which can facilitate the release of glutamate (Mansvelder and McGehee 2000; Schilström et al. 2000; Jones and Wonnacott 2004). When presynaptic nicotine is paired with postsynaptic depolarisation, long-term potentiation of glutamatergic transmission results in vitro (Mansvelder and McGehee 2002). The coupling of α7 nAChR to CICR (see Sect. 2.2.1) provides a potential mechanism for presynaptic facilitation mediated

by these receptors (Emptage et al. 2001; Collin et al. 2005; Sharma et al. 2008). In response to a bolus of nanomolar nicotine in vivo, it is predicted that the sustained activation of presynaptic α7 nAChRs to facilitate glutamate release coincides with the transient activation of somatodendritic heteromeric nAChRs, which thereby contribute to the postsynaptic depolarisation (McKay et al. 2007). Decreased GABAergic input (attributed to long-term depression at GABAergic synapses, instigated by transient activation of α4β2 nAChRs; Mansvelder and McGehee 2002) would also facilitate depolarisation of dopamine neurones (McKay et al. 2007).

Strengthening of glutamatergic synapses within the VTA is also observed in response to other abused drugs such as cocaine and amphetamine. Importantly, this cannot be induced by non-addictive drugs such as fluoxetine or carbamazepine (Saal et al. 2003). Hence, synaptic neuroadaptation at excitatory synapses is an important key step in the development of addiction (Kauer and Malenka 2007).

2.2.4 Somatodendritic Signalling and the Regulation of Gene Expression

The increases in intracellular Ca^{2+} that accompany the activation of somatodendritic nAChRs can facilitate the engagement of Ca^{2+}-sensitive proteins (notably kinases, but also phosphatases) and modulation of intracellular signalling cascades. In addition to short-term local changes, such as nAChR phosphorylation that can modify receptor function (Eilers et al. 1997), transduction of signals to the nucleus can exert longer-term changes by influencing gene transcription. This has been most clearly demonstrated in cultured neurones or cell lines: activation of somatic nAChRs by nicotine results in increases in the transcriptional regulator phospho-CREB (cyclic AMP response element-binding protein) and immediate early gene c-Fos (Hu et al. 2002). The phosphorylation of CREB is downstream of extracellular signal-regulated kinase ERK1/2 that is activated following Ca^{2+} entry via α7 nAChRs (Hu et al. 2002; Bitner et al. 2007), although some studies dispute the involvement of α7 nAChRs (Nakayama et al. 2001; Steiner et al. 2007). In mice, acute administration of a low dose of nicotine ($0.4\,mg\,kg^{-1}$) increased phospho-ERK1/2 immunoreactivity in the NAc, prefrontal cortex and some other regions innervated by the mesolimbic dopamine pathway. A similar pattern of response was elicited by other drugs of abuse, but not by non-addictive drugs (Valjent et al. 2004).

In catecholaminergic PC12 cells, nicotine-induced activation of CREB is coupled to increased transcription of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine and noradrenaline (Gueorguiev et al. 2006). This has been attributed to activation of α7 nAChRs and CICR (Gueorguiev et al. 2000), although activation of ERK1/2 did not appear to be necessary (Gueorguiev et al. 2006). In vivo, upregulation of tyrosine hydroxylase protein in response to oral nicotine in the drinking water (see Sect. 3.1) has been demonstrated in mice; enzyme levels were increased in the amygdala and prefrontal cortex, but not in VTA or NAc (Brunzell et al. 2003). The ability of nicotine to enhance catecholaminergic transmission by increasing the amount of rate-limiting enzyme is appealing in terms of nicotine reinforcement, and a polymorphism in this enzyme has been associated with reduced

nicotine dependence (Anney et al. 2004). However, there was no consistent correlation between changes in tyrosine hydroxylase, ERK1/2 and phospho-CREB in the mouse study (Brunzell et al. 2003). This could reflect the multiple roles of ERK1/2 in coordinating diverse signals to regulate multiple effectors (Girault et al. 2007; Zhai et al. 2008).

Particular attention has been given to the transcription of immediate early genes such as c-Fos. As transcriptional regulators, these downstream effectors of intracellular signalling cascades can play a pivotal role in switching on or off genes critical for the development of neuronal adaptations. c-Fos mRNA and protein expression in rat NAc, amygdala and other reward-related regions are increased in response to acute nicotine administration in vivo (Salminen et al. 1999; Shram et al. 2007). Moreover, nicotine injection directly into the VTA increased c-Fos-like immunoreactivity in the NAc (Panagis et al. 1996). The latter study is important because it clearly demonstrates that immediate early gene regulation does not occur in dopaminergic neurones bearing nAChRs in the VTA, but in the postsynaptic neurones (probably GABAergic) in the terminal field. Thus, although cellular studies can reveal a series of signalling events culminating in gene expression, in vivo responses are more complex and may be the result of increased transmitter release activating postsynaptic neurones. In this regard, it is noteworthy that nicotine-evoked increases in phosphoERK1/2 are blocked by D1 receptor antagonists (Valjent et al. 2004).

Microarray technology to screen for changes in gene expression offers a means of identifying novel targets. A comparison of the transcriptomes of the SH-SY5Y neuroblastoma cell line with and without treatment with a high concentration of nicotine (1 mM) for 1 hour identified 14 genes with altered levels of expression (Dunckley and Lukas 2003). The changes were mostly nAChR-mediated as they were prevented by co-administration of nicotinic antagonists. The genes affected include transcription factors, RNA binding proteins and plasma membrane proteins; their physiological roles and implications for nicotine dependence are presently unclear.

3 Effects of Chronic Nicotine

Nicotine absorption through inhalation of tobacco smoke is the most widespread mode of nicotine consumption. Regular cigarette smoking leads to complex kinetics of plasma nicotine levels, and the concentration and time-course of nicotine reaching central nAChRs remains controversial (Rose et al. 1999; Hukkanen et al. 2005). Modelling this phenomenon in animals for research purposes is challenging. Humans are the only animals that will voluntarily inhale; exposure of animals to cigarette smoke has been attempted (e.g. using nose cones for forced inhalation), but such methods are highly stressful and confound the validity of this approach. In the light of the Surgeon General's Report in 1990, research has concentrated on the effects of nicotine. Several methods of nicotine delivery have been developed to model various aspects of nicotine intake in order to evaluate ensuing

molecular and cellular changes in the brain. This has been comprehensively reviewed by Matta et al. (2007). The main issues, which will be briefly addressed below, are dose, route, timing and non-contingent administration.

3.1 Animal Models of Nicotine Administration

Once- or twice-daily injections deliver a single bolus of drug that reproduces, to some extent, the predicted increases in plasma nicotine that accompany consumption of a cigarette. Typical behaviourally effective doses of nicotine free base range from 0.2 to 0.8 mg kg^{-1} in rats, with higher doses employed in mice (up to 2.0 mg kg^{-1}) to compensate for the increased rate of nicotine metabolism in mice (Hukkanen et al. 2005). When injected subcutaneously, nicotine rapidly penetrates the bloodstream, although peak levels (15 min post-injection; Turner 1975) are achieved more slowly than when inhaling nicotine (~10 s; Rose et al. 1999; Hukkanen et al. 2005). Hence, to avoid desensitisation of nAChRs by the initial lower levels of nicotine that may result from the slower elevation of plasma concentrations following injection, nicotine is typically administered at higher doses than one would expect.

Injections fail to recapitulate the repetitive nature of nicotine delivery arising from smoking a single cigarette over about 15 min, or the effect of smoking multiple cigarettes. This has been attempted in mice using in-dwelling catheters for nicotine delivery via an automated system (Robinson et al. 1994). This technology is well established for nicotine self-administration in rats (see chapter by Balfour, this volume), which combines frequent low doses with voluntary lever-pressing. Shoaib and Stolerman (1999) showed that rats given the opportunity to lever-press for intravenous nicotine delivery (0.03 or 0.06 mg kg^{-1} delivered in ~1 s) achieved plasma levels of nicotine that are comparable to those observed in smokers (40–120 ng ml^{-1}). Recently, it has been argued that lower doses (corresponding to 1–2 typical cigarette puffs; 3 µg kg^{-1}) given over a slower time-course (30 s) are preferentially self-administered, and that the speed of infusion influences the interactions with dopamine systems (Sorge and Clarke 2007). However, self-administration has rarely been used or adapted for molecular and cellular studies.

During the day, assuming regular cigarette consumption and the slow release of nicotine from body tissues, plasma nicotine reaches steady-state levels of approximately 20–70 ng ml^{-1} (Gourlay and Benowitz 1997; Russell 1990). Osmotic minipumps provide a constant, slow delivery of nicotine (0.1–10 µl h^{-1}) over a modest period of time (1–4 weeks, depending on the model of minipump employed) to mirror these sustained concentrations: 2–4 mg kg^{-1} per day in rats achieves plasma nicotine concentrations of 20–50 ng ml^{-1} (Barik and Wonnacott 2006; Rowell and Li 1997; Sanderson et al. 1993). Once implanted, there is minimal animal handling and stress. The main limitation is the constant infusion of nicotine, even during non-active periods, whereas overnight abstinence is a key feature of human consumption.

An alternative method of long-term administration that circumvents this issue is nicotine administration via drinking water. This limits consumption to the active phase, and can encompass some self-control of nicotine consumption by the animal (depending on whether a two-bottle choice is available). However, the bitter taste of nicotine makes it unpalatable, causing reduced water intake and loss of body weight. The taste must be disguised by additives such as saccharin, which introduces another parameter that must be controlled for. Typical concentrations of nicotine in drinking water are 20–30 $\mu g\,ml^{-1}$, and are increased progressively by 50 μg increments per week over several weeks of treatment. Plasma nicotine concentrations achieved by this route in primates and rodents are modest (10–35 $ng\,ml^{-1}$; Pietilä and Ahtee 2000; Quik et al. 2006; Rowell et al. 1983), due to first-pass metabolism by the liver.

The methods discussed above have been widely used to assess the effect of either continuous or intermittent nicotine on nAChR functions and brain biochemistry (Matta et al. 2007). For models of nicotine withdrawal, see the chapter by Malin in this volume. Given the intrinsic advantages and limitations of each approach, the non-contingent nature of most administration regimes and the absence of associated cues, it is important that these paradigms are not assumed to model "tobacco addiction" per se. Sometimes, the experiments are conducted in concert with behavioural measures (e.g. precipitation of withdrawal with somatic signs), which give more credibility to the assertion that a state of nicotine dependence has been achieved (Kenny and Markou 2005).

3.2 Changes in Nicotine-Evoked Neurotransmitter Release

As described in Sect. 2.2.3, nAChRs can modulate the release of numerous neurotransmitters. Although prolonged nicotine exposure can perturb these neurotransmitter systems, the functionality of nAChRs following such treatments remains a debated issue. Due to the predominance of the dopaminergic hypothesis of addiction, attention has focussed on the nicotinic modulation of striatal/accumbal dopamine release. However, the complex interplay of glutamate and GABA inputs (Fig. 3) and the contribution of the noradrenergic and serotoninergic systems to addiction (Done et al. 1992; Vorel et al. 2001; Weinshenker and Schroeder 2007) demands a broader view of nicotine's effects.

In ex vivo studies in which transmitter release is measured in synaptosome or slice preparations from animals treated with nicotine in vivo, no change (Grilli et al. 2005), decreased (Grady et al. 1997) and increased (Yu and Wecker 1994) striatal [^{3}H]dopamine release has been reported. A similar picture has been observed for nAChR-evoked hippocampal [^{3}H]noradrenaline release, with unchanged (Barik and Wonnacott 2006), decreased (Grilli et al. 2005) and increased (Jacobs et al. 2002) responses. As tissue is extensively washed prior to nAChR stimulation, these discrepancies are unlikely to reflect differences in nAChR desensitisation due

to residual nicotine but are probably due to differences in experimental procedures, including the dose of nicotine employed, the route and frequency of administration, and the brain tissue preparation used (synaptosomes or slices). In at least two studies, an increase in noradrenaline release during the withdrawal phase has been noted (Gaddnas et al. 2000; Barik and Wonnacott 2006).

With respect to in vivo noradrenaline release, several studies report increases in response to nicotine challenge after chronic administration, consistent with a sensitised response. Sharp and co-workers demonstrated that rats self-administering nicotine in an unlimited access paradigm exhibited markedly increased levels of endogenous noradrenaline in the hypothalamic paraventricular nucleus (Fu et al. 2001) and amygdala (Fu et al. 2003). Also, in rats that received a daily nicotine injection ($0.4\,mg\,kg^{-1}$) for 5 days, noradrenaline release in the ventral hippocampus was enhanced in response to a subsequent nicotine challenge (Benwell and Balfour 1997).

Responses in the dopamine system are more complex (see chapter by Balfour, this volume). Repeated nicotine injections resulted in enhanced extracellular DA levels in the NAc (Benwell and Balfour 1992, 1997), but not in the striatum (Benwell and Balfour 1997). Analysis of the precise placement of dialysis probes has revealed differential responses to drugs of abuse, including nicotine, between the NAc core (ventral striatum) and shell (Di Chiara 2002; Balfour 2004; Wonnacott et al. 2005; see chapter by Balfour, this volume). Moreover, the sensitised neurotransmitter responses observed in the hippocampus and NAc were markedly attenuated if rats received a constant infusion of a low level of nicotine (Benwell and Balfour 1997). Thus, transient peaks of nicotine appear capable of sensitising some brain pathways with respect to catecholamine release, but the responses may be mitigated by lower sustained plasma concentrations, possibly due to desensitisation. The extent that presynaptic nAChRs contribute to this process in vivo is unclear; presynaptic $\alpha 7$ nAChRs on glutamatergic afferents to the VTA merit attention as potential mediators of sensitisation (see Sect. 2.2.2).

3.3 Alterations in Gene and Protein Expression

What are the molecular and cellular changes that confer a state of dependence in response to abused drugs? In addressing this question, several studies have focussed on the ERK1/2 signalling cascade for linking nAChR activation by nicotine to long-term cellular changes (see Sect. 2.2.4). This pathway modulates associative learning processes and reward, and a growing body of evidence indicates that drugs of abuse hijack these physiological processes (Berke and Hyman 2000; Hyman and Malenka 2001; Zhai et al. 2008). Indeed, complex changes in ERK1/2 and CREB were observed following chronic oral nicotine administration in mice (Brunzell et al. 2003).

Downstream targets of ERK1/2 and CREB include immediate early genes, such as c-Fos, FosB and Zif268, which are expressed in response to drugs of abuse

(Lee et al. 2005; Valjent et al. 2006; Zhang et al. 2006), including chronic nicotine (Pagliusi et al. 1996; Nisell et al. 1997; Soderstrom et al. 2007). A long-lived truncated isoform of the FosB protein, ΔFosB, has been shown to accumulate within the striatum of rats treated repeatedly with either cocaine or nicotine (Hope et al. 1994; Pich et al. 1997; Nestler 2001). ΔFosB persists for several weeks in the brain, and represents an example of a sustained molecular change initiated by drug experience, although it cannot solely account for the perseverance of drug dependence.

Microarray technology has been employed to compare changes in gene expression in brain reward areas of rodents exposed to nicotine (Konu et al. 2001; Li et al. 2004; Vadasz et al. 2007; Wang et al. 2008). Analysis of such studies in mice is complicated by strain differences, with implications for assessing transgenic animals (Kedmi and Orr-Urtreger 2007; Vadasz et al. 2007; Wang et al. 2008). Putative gene candidates arising from these studies are functionally diverse, and include examples involved in cell signalling, cell structure and gene regulation (such as zinc-finger DNA binding proteins). Although these studies have not clearly identified one (or more) common gene (or genes) that could underpin the addictive properties of nicotine, they all pinpoint genes encoding components of intracellular trafficking pathways. This is interesting in view of the upregulation of nAChRs following chronic nicotine treatment (see Sect. 3.4).

From the brain microarray studies following in vivo nicotine administration, it is not clear which nAChR subtypes are involved in initiating the observed changes in gene expression. Responses in SH-SY5Y cells suggest a complex interplay between α7 and non-α7 nAChR signalling (Dunckley and Lukas 2006). In this cell line, the lack of correspondence in the pattern of genes modified following either 1 h (Dunckley and Lukas 2003) or 24 h (Dunckley and Lukas 2006) of nicotine treatment highlights the complexity and time-dependency of the changes triggered by prolonged nicotine exposure. Indeed, in vivo acute and chronic nicotine treatments elicit distinct patterns of changes in total and phosphorylated proteins such as ERK1/2 and CREB (Brunzell et al. 2003), as well as in their targets (Salminen et al. 1999; Nuutinen et al. 2007), and withdrawal can precipitate another pattern of changes.

Recently, epigenetic mechanisms (responsible for "permanent" modifications of gene expression) are gaining attention in the field of abused drugs (Tsankova et al. 2007). Repression or enhancement of gene transcription is tightly linked to the state of chromatin compaction, and chromatin remodelling is achieved by complex post-translational modifications of histones. For example, phosphorylation of histone H3 is regulated by the ERK1/2 pathway (Girault et al. 2007). Although there is increasing evidence that cocaine triggers chromatin remodelling (Brami-Cherrier et al. 2005; Kumar et al. 2005; Tsankova et al. 2007), there is presently no report of nAChR-elicited covalent histone alterations in the brain in response to nicotine. Hence, further work is required to determine to what extent the persistence of nicotine-addicted behaviours involve chromatin remodelling and which nAChRs and signalling cascades are involved.

3.4 Mechanisms and Consequences of nAChR Upregulation

3.4.1 Paradoxical Effects of Prolonged Nicotine Exposure

The generation of nicotinic radioligands (including [^{3}H]nicotine, [^{3}H]epibatidine, ^{125}I-α Bgt, [^{3}H]MLA and ^{125}I-α conotoxinMII) facilitated analysis of the distribution and density of nAChR binding sites in the brain (see chapter by Collins et al., this volume). Equilibrium binding experiments to determine the total number of binding sites (B_{max}) and apparent affinity for ligand (K_d) require long incubation times (typically, hours). In the case of agonist ligands, this prolonged exposure promotes nAChR desensitisation (Fig. 1a), hence K_d values will reflect the affinity of the desensitised nAChR for the agonist in question (Lippiello et al. 1987).

Early ligand binding studies indicated that chronic nicotine treatment increased the number of brain nAChR binding sites (Marks et al. 1983; Schwartz and Kellar 1983), and this was recapitulated in a comparison of brain tissue from smokers and non-smoker controls (Benwell et al. 1988; Wonnacott 1990). Two decades later, the ability of prolonged exposure to nicotine to upregulate nAChR binding sites is a robust observation, both in vivo, in numerous species (including mice, rats, monkeys and humans) and in vitro, in various homologous and heterologous expression systems (Gentry and Lukas 2002). A large variety of other nicotinic agonists, as well as some antagonists and compounds that enhance cholinergic transmission (such as acetylcholinesterase inhibitors), also increase numbers of nAChRs, whereas numbers of muscarinic receptors are unaffected by prolonged nicotine exposure (Lapchak et al. 1989; Sanderson et al. 1993). The phenomenon reflects an increase in B_{max}, with no change in receptor affinity, K_d (Wonnacott 1990). However, the mechanisms underlying this paradoxical effect, as well as its functional significance and role in addiction, are still debated.

3.4.2 Mechanisms of nAChR Upregulation

In the quest to understand the mechanisms of upregulation, many studies have focussed on α4β2* nAChRs as they represent the most abundant high-affinity nAChR binding sites in the brain and are implicated in mediating the reinforcing properties of nicotine (Picciotto et al. 1998; Maskos et al. 2005). It is generally agreed that the reversible phenomenon of upregulation is an intrinsic feature of nAChRs ("receptor-autonomous"), requiring conserved cellular processes common to neuronal and non-neuronal systems ("cell-autonomous") (Nashmi and Lester 2007). Upregulation of nAChR binding sites is not accompanied by any change in mRNA; therefore, the mechanism is post-transcriptional. It was originally posited that upregulation is a response to nAChR desensitisation or longer-term inactivation, due to nicotine acting at cell surface receptors (Dani and Heinemann 1996). However, comparison of different nicotine doses and administration schedules in rats showed that continuous administration and twice-daily infusions increased brain nAChR numbers, whereas more frequent intermittent schedules did not, despite achieving

comparable blood and brain nicotine levels (Rowell and Li 1997). In cultured cells in vitro, the concentration dependence of upregulation of α4β2 nAChRs matches neither the concentration dependence of nAChR activation nor desensitisation, for any given agonist (Whiteaker et al. 1998). Moreover, upregulation has also been provoked by both competitive (DHβE, MLA) and non-competitive (mecamylamine) antagonists (Peng et al. 1994; Gopalakrishnan et al. 1997; Whiteaker et al. 1998; Molinari et al. 1998; Gentry and Lukas 2002).

The agonist radioligands [^{3}H]nicotine and [^{3}H]epibatidine, which are most commonly used to monitor nAChR numbers, freely cross lipid bilayers. Therefore, they do not discriminate between intracellular and surface nAChRs and the majority of the increase in high-affinity binding sites appears to be intracellular (Whiteaker et al. 1998; Vallejo et al. 2005). It is now appreciated that nicotine can accumulate within cells and a new consensus is emerging that supports an intracellular action of nicotine that enhances assembly and/or maturation of nAChRs (Nashmi et al. 2003; Darsow et al. 2005; Kuryatov et al. 2005; Sallette et al. 2004, 2005; Nashmi and Lester 2007; Fig. 4).

The elegant studies of Lester and colleagues have exploited fluorescently tagged α4 and β2 subunits to facilitate analysis of subunit interactions using fluorescence resonance energy transfer (FRET). Chronic nicotine treatment (1 μM, 24 h) increased the FRET signal, indicative of increased nAChR assembly (Nashmi et al. 2003). These results are consistent with the study of Sallette et al. (2004) that identified an extracellular microdomain close to the agonist binding site of the β2 subunit that is crucial for nicotine-induced nAChR upregulation. Subsequent metabolic labelling and immunoprecipitation studies from the same group indicated the endoplasmic reticulum as the site where nicotine acts as a "maturational enhancer" (Sallette et al. 2005). Comparison of the localisation of immunolabelled nAChR subunits with markers that define various intracellular compartments also identified the site of the action of nicotine "at the level of, or prior to, the ER" (Darsow et al. 2005). The amount of mature β2 subunits detected with conformationally dependent antibodies increased following chronic nicotine, indicating that nicotine induced a conformational change in the β2 subunit that may favour or accelerate nAChR assembly (Sallette et al. 2005). This is also compatible with the change in stoichiometry of functional cell-surface nAChRs in heterologous expression systems, from $(\alpha4)_3(\beta2)_2$ to $(\alpha4)_2(\beta2)_3$, in response to chronic nicotine (Nelson et al. 2003; Moroni et al. 2006). Such a change could be driven by an increase in correctly folded β2 subunits. As the $(\alpha4)_2(\beta2)_3$ stoichiometry displays increased sensitivity to ACh, this switch could have physiological consequences. If this stoichiometry also engenders higher affinity for nicotine, it reconciles the claim that upregulation reflects the stabilisation of α4β2 nAChRs in a high-affinity state (Vallejo et al. 2005).

The most parsimonious model for upregulation that accommodates current information suggests that nicotine binding to immature subunits enhances receptor assembly by provoking quaternary structure rearrangements (that might result in altered subunit stoichiometry), leading to accelerated maturation of nAChRs and a net

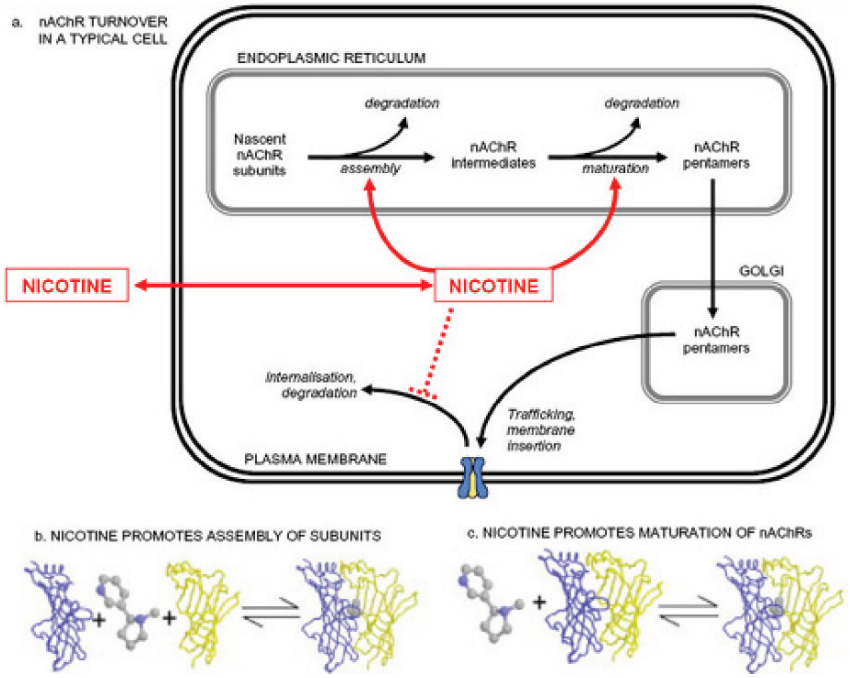

Fig. 4 Current model for nicotine upregulation of α4β2 nAChRs. **a** Schematic of a cell indicating major steps in the lifecycle of a nAChR. Nicotine accumulates within the cell. Within the endoplasmic reticulum, nicotine binds to nAChR subunits to facilitate assembly, or binds at the interface of an αβ subunit pair to enhance maturation of a pentameric nAChR (Sallette et al. 2004, 2005). The strong influence of nicotine on maturation of the β2 subunit might also favour a change in nAChR stoichiometry, from $(\alpha4)_3(\beta2)_2$ to $(\alpha4)_2(\beta2)_3$ (Moroni et al. 2006). These actions could result in an increase in the membrane insertion of competent nAChRs. The possibility of an additional action of nicotine to impede nAChR turnover or degradation is indicated by the *dotted line*. **b** Binding of nicotine to the extracellular domain of unassembled nAChR subunits facilitates assembly. **c** Binding of nicotine at an αβ interface facilitates maturation of a pentameric nAChR. Items **b** and **c** adapted from Nashmi and Lester (2007), with permission from Elsevier

increase in high-affinity receptors (Fig. 4). Additional influences, such as decreased rate of turnover of membrane nAChRs (Kuryatov et al. 2005; Rezvani et al. 2007), may also contribute.

3.4.3 Differential Upregulation of nAChR Subtypes

The extent of upregulation varies with nAChR subtype and is typically much greater in cell lines than in native tissues after in vivo exposure to nicotine. The β2-containing nAChRs display the highest level of upregulation (Xiao and Kellar 2004), reflecting differences in the interface between adjacent α and β subunits, with respect to β2 versus β4 subunits (Sallette et al. 2004). Interestingly, inclusion of the

α5 subunit (which appears to associate exclusively with α4 and β2 subunits in native nAChRs) prevents upregulation in vivo (Mao et al. 2008). Thus, the intrinsic properties of each subunit influence the efficacy of upregulation, and this could contribute to regional differences in the extent of upregulation observed in response to nicotine treatment in vivo (Pauly et al. 1991).

In contrast to α4β2* nAChRs, measurements of α6β2 ∗ nAChRs using quantitative autoradiography with ^{125}I-αconotoxinMII show these receptors to be unchanged or decreased in number in the dopaminergic systems of both rodents and monkeys after chronic nicotine administration by a variety of routes (Lai et al. 2005; McCallum et al. 2006; Mugnaini et al. 2006). The α6β2* nAChRs also bind [^{3}H]epibatidine and, when measured using this radioligand in transfected HEK cells such nAChRs, are upregulated in response to chronic nicotine (Tumkosit et al. 2006; Walsh et al. 2008). One interpretation that reconciles these disparate observations is that the impermeable antagonist ^{125}I-αconotoxinMII does not label the same populations or states of the receptor as the permeable agonist [^{3}H]epibatidine (Walsh et al. 2008); for example, the αconotoxin might only bind to fully mature nAChRs in the plasma membrane, whereas epibatidine labels multiple high-affinity states during the assembly and maturation process.

The upregulation of α7 nAChRs, identified by binding of the antagonist snake toxin ^{125}I-αBgt, suggests that a net increase in these receptors does occur in response to nicotine (Pauly et al. 1991; Rasmussen and Perry 2006). Additional or different mechanisms contribute to the upregulation of α7 nAChRs, compared with heteromeric nAChR subtypes (Ridley et al. 2001; Massey et al. 2006; Nuutinen et al. 2006). Upregulation of α7 nAChRs occurs at higher nicotine concentrations than required to increase α4β2 nAChRs (Pauly et al. 1991; Kawai and Berg 2001); therefore, the association of α7 nAChR upregulation with nicotine dependence is uncertain, with mixed reports in rodents treated with smokers' levels of nicotine (Sanderson et al. 1993; Mugnaini et al. 2006; Rasmussen and Perry 2006). There are no reports of the density of α7 nAChR binding sites in the brains of human smokers, although α7 nAChR immunoreactivity in astrocytes is reported to be decreased in smokers (Teaktong et al. 2004).

3.4.4 Functional Status of Upregulated nAChRs

Although it has been widely assumed that the upregulation of high affinity nAChRs by nicotine must be central to nicotine addiction, direct evidence for this is lacking. Indeed, the functional status of upregulated nAChRs is still controversial (for a review, see Gentry and Lukas 2002). Addressing this question is hampered by the presence of the nicotine necessary to provoke upregulation (predicted to desensitise nAChRs), so adequate time for washout of drug (intracellular as well as extracellular) must be allowed. Direct measurement of nAChR activation using electrophysiology or rubidium ($^{86}Rb^+$) efflux assays can give different answers from those derived from downstream readouts, such as calcium fluorimetry or [^{3}H]neurotransmitter release techniques. Some studies have demonstrated a loss

of functional responses governed by various nAChR subtypes, including α7 and α4β2, following chronic nicotine exposure (Marks et al. 1993; Eilers et al. 1997; Olale et al. 1997; Ridley et al. 2002), while others have shown enhanced functionality of both α7 and non-α7 nAChRs (Buisson and Bertrand 2001; Nashmi et al. 2003; Sokolova et al. 2005). Surprisingly, Buisson and Bertrand (2001) found that ACh-evoked currents, albeit of small amplitude, could be recorded when K-177 cells expressing human α4β2 nAChRs were still bathed with nicotine, whereas other studies have emphasised the occurrence of long-term functional inactivation in response to sustained nicotine exposure (Eilers et al. 1997). In heterologous expression systems at least, it has been proposed that upregulation reflects the stabilisation of α4β2 nAChRs in a high affinity state that is more readily activated (Vallejo et al. 2005), perhaps reflecting a switch in the stoichiometry of α and β subunits, as discussed in Sect. 3.4.2.

Despite numerous studies, it remains uncertain what role, if any, upregulated nAChRs would have in the induction or maintenance of nicotine dependence. nAChR upregulation can be achieved in animals by paradigms that have little addiction liability in humans: nicotine delivery by osmotic minipumps best equates with transdermal nicotine delivery in humans. However, it is plausible that upregulated numbers of nAChR may play a role during nicotine withdrawal when exposure to nicotine is eliminated. Recovery of receptor function would generate enhanced responses to endogenous levels of ACh that could contribute to withdrawal symptoms or craving. Indeed, after 7 days of abstinence from smoking, human smokers subjected to single-photon emission computed tomography (SPECT) showed higher levels of β2* nAChRs that correlated with the urge to smoke to relieve withdrawal symptoms (but not with the severity of either dependence or withdrawal) (Staley et al. 2006).

4 Concluding Remarks

nAChRs are the primary conduit for the effects of nicotine on the CNS. Detailed understanding of the molecular and cellular mechanisms associated with these receptors has emerged in recent years and reveals several levels of complexity. The molecular diversity of nAChR subunits creates a wide array of subtypes differing in their sensitivity to nicotine and in their intrinsic properties. The inclusion or omission of a single subunit can have significant impact; for example, the effect of the α5 subunit on nAChR function and upregulation. The differential cellular and subcellular localisation of these nAChRs is very pertinent to the propensity of nicotine to induce and sustain dependence. The mesocorticolimbic dopamine system and associated GABAergic and glutamatergic inputs are endowed with a rich repertoire of nAChRs, whose functional significance is only partly understood. Mechanistically, nAChRs can exist in multiple states and the balance between activation and desensitisation (which varies with nAChR subtype) is considered critical for the reinforcing properties of nicotine. At a cellular level, the ability of nAChRs to elicit

significant changes in intracellular Ca^{2+} facilitates the engagement of downstream signalling pathways, allowing both short-term changes, such as presynaptic plasticity, and longer-term influences realised through modification of gene transcription, and perhaps involving epigenetic alterations. Unravelling these contributions in the context of tobacco smoking – a complex nicotine delivery profile associated with powerful reinforcing cues – remains a challenge.

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The Neuronal Pathways Mediating the Behavioral and Addictive Properties of Nicotine

David J.K. Balfour

Contents

Abstract This chapter considers the neurobiological mechanisms that are thought to mediate the reinforcing or rewarding properties of nicotine. It focuses on the data (derived principally from studies with experimental animals) showing that nicotine, like other drugs of dependence, stimulates the mesolimbic dopamine (DA) neurones that project to the nucleus accumbens and that these effects play a pivotal role in the biology underlying nicotine dependence. The reinforcing or rewarding properties of nicotine are thought to be associated particularly with the increase in DA overflow evoked in the shell subdivision of the accumbens. However, behavioural studies suggest that these properties of nicotine in experimental animals do not seem to be sufficiently potent to explain the powerful addiction to tobacco experienced by most habitual smokers. This chapter also considers the biological mechanisms that

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mediate the effects of cues and stimuli associated with the presentation of nicotine, which are thought to contribute significantly to the powerful addictive properties of tobacco smoke.

1 Introduction

It is now widely accepted that nicotine is the principal addictive component of tobacco smoke and that many habitual smokers find it difficult to quit the habit because they have become dependent upon the nicotine they inhale in the smoke. Drugs of dependence generally exert two effects that contribute to their addictive properties. They have reinforcing or rewarding effects, which an addicted individual, it is assumed, finds so powerful that the drive to re-experience these effects comes to dominate the behavioural repertoire of the addict. Chronic or repeated exposure to addictive drugs also often results in biological changes within the brain and elsewhere in the body, which cause an aversive abstinence syndrome when the drug is withdrawn precipitously. Thus, addicted individuals may continue taking the drug in order to avoid experiencing the abstinence syndrome. Studies with experimental animals suggest that nicotine exerts both of these effects. The behavioural features of nicotine dependence are considered in detail elsewhere in this handbook. This chapter will focus on some of the neurobiological mechanisms that are thought to mediate the addiction to nicotine.

There is little evidence that nicotine is abused in its pure form, but is taken as a constituent of tobacco, most commonly being inhaled in tobacco smoke. Indeed, studies with experimental animals suggest that nicotine is a relatively weak reinforcer when compared with other drugs of dependence, such as amphetamine, cocaine or morphine (Caggiula et al. 2001; Donny et al. 2003). Thus, the reinforcing properties of nicotine *per se* would not seem to provide an adequate explanation for the powerful addictive properties of tobacco smoke (Balfour 2004). One possible explanation, proposed by Le Foll and colleagues, is that the rewarding or reinforcing properties of nicotine are more potent in higher animals with more complex cognitive skills (Le Foll et al. 2007). Studies in both human smokers and experimental animals suggest that the addictive properties of tobacco depend upon the context in which it is used and, especially, on the sensory stimuli associated with its delivery (Rose et al. 1993; Brauer et al. 2001; Caggiula et al. 2001; Donny et al. 2003; Palmatier et al. 2006; Balfour 2004). It is also important to remember that tobacco smoke contains other components that may enhance the addictive properties of nicotine. For example, tobacco contains compounds that inhibit monoamine oxidase and might be expected to enhance the effects of monoamines, especially dopamine (DA), whose release is stimulated by nicotine (Fowler et al. 2003). Other studies suggest that the acetaldehyde present in tobacco smoke may enhance the addictive potential of the smoke (Talhout et al. 2007). Thus, any explanation for the powerful addictive properties of tobacco must take account of these facts when seeking to describe the neurobiology underlying the addiction to tobacco.

2 The Role of Mesolimbic Dopamine

For the last two decades, the mesolimbic dopamine (DA) hypothesis of dependence has dominated thinking in relation to the neurobiological mechanisms that underpin the addiction to drugs (e.g. Wise and Bozarth 1987). Several lines of evidence provide strong support for the hypothesis. Microdialysis studies have shown that most, if not all, drugs of dependence elicit a preferential increase in DA release from the mesolimbic neurones that project to the nucleus accumbens (e.g. Di Chiara and Imperato 1988; Di Chiara 2002). Other studies have shown that experimental animals will learn to stimulate electrodes located in the principal DA pathways of the brain, using an intracranial self-stimulation paradigm of reward. The threshold current (the brain reward threshold) required to evoke this response is diminished if the animals are pretreated with drugs such as nicotine, amphetamine or cocaine, which enhance DA overflow in the nucleus accumbens (Bozarth et al. 1998; Pidoplichko et al. 1997; Lin et al. 2000; Kling-Petersen et al. 1994). These observations have encouraged speculation that the mesolimbic DA system of the brain may contribute to a "reward system", which responds to pleasurable stimuli (Wise and Bozarth 1987; Wise 2004). Other studies, employing intravenous self-administration as a measure of the reinforcing properties of nicotine, have shown that preferential lesions of the DA projections to the nucleus accumbens attenuate responding for nicotine (Corrigall et al. 1992). Lesions of these projections also attenuate responding for the other principal psychostimulant drugs of dependence, amphetamine and cocaine (Lyness et al. 1979; Roberts and Koob 1982). It seems reasonable to conclude, therefore, that the reinforcing properties of nicotine depend upon its ability to stimulate DA release in the nucleus accumbens, the principal terminal field of the mesolimbic DA system.

The nucleus accumbens is a complex structure that incorporates two principal subdivisions: a central core and a shell that surrounds the core on its medial and ventral sides. These two subdivisions are anatomically distinct and are thought to subserve different functions (Heimer et al. 1991; Zahm and Brog 1992). The accumbal shell appears to form part of an extended amygdala and, thus, is part of the limbic system. The neurones of the accumbal core resemble more closely those of the dorsal striatum and send major projections to areas of the brain concerned with the control of motor function. Rodd-Henricks and colleagues (2002) have shown that rats can be trained to self-administer microinjections of cocaine directly into the accumbal shell, whereas the animals will not self-administer the drug through cannulae targeted at the accumbal core. Sellings and Clarke (2003) used a conditioned place preference paradigm to explore the role of the DA projections to the accumbal core and shell in the rewarding properties of amphetamine. They reported that preferential lesions of the DA projections to the medial accumbal shell attenuated the rewarding properties of amphetamine when measured using the place preference paradigm. In contrast, lesions of the DA projections to the accumbal core attenuated the locomotor stimulant response to the drug. Similar experiments have yet to be performed using nicotine. Nevertheless, the data when taken together provide support for the conclusion that stimulation of DA release in the shell subdivision

of the accumbens mediates the rewarding or reinforcing properties of psychostimulant drugs, whereas increased DA release in the core subdivision mediates their locomotor stimulant properties (Balfour 2004). There is, however, evidence that the DA projections to other neuroanatomical sites, such as the olfactory tubercle, also contribute to the reinforcing properties of cocaine (Ikemoto 2003). Furthermore, a more recent study, designed to explore the neuroanatomical sites responsible for the behavioural responses to the stimulant methylphenidate, has provided further evidence for the conclusion that the locomotor stimulant properties of psychostimulant drugs are related to increased DA release in the core subdivision of the accumbens. The rewarding properties of methylphenidate, again measured using the conditioned place preference paradigm, may depend upon increased DA release in the anterior medial olfactory tubercle (Sellings et al. 2006). These observations emphasise the need to be cautious when attributing the reinforcing and locomotor stimulant properties of nicotine solely to events in the two principal subdivisions of the nucleus accumbens.

It is also important to acknowledge that the results of some studies that have sought to explore the role of DA pathways in reward and reinforcement have generated results that cast doubt on a simple relationship between increased DA release in mesolimbic regions of the brain and the reinforcing properties of drugs of abuse. Pettit and colleagues (1984) showed that in rats trained to lever-press for heroin, selective lesions of the DA projections to the limbic forebrain had no significant effects on the established responding for heroin but attenuated responding for cocaine. Similar findings have been reported by Gerrits and Van Ree (1996), who concluded that their results argued against a critical role of the accumbal DA projections in the motivational mechanisms underpinning drug abuse. A subsequent study by Rocha and colleagues (1998) showed that transgenic mice lacking the neuronal DA transporter, the principal neural target for cocaine, can still learn to respond for cocaine in a manner similar to that found for wild-type animals. The authors concluded that increased DA overflow alone could not explain the reinforcing properties of the drug. More recently, Cannon and Palmiter (2003) used a transgenic mouse strain that lacked tyrosine hydroxylase, and therefore the ability to form DA, to show that animals can still learn a task reinforced by a sucrose reward in the absence of DA. These data suggest that, in addition to DA-dependent mechanisms, there must be other DA-independent pathways that allow acquisition of reward-reinforced behaviours.

A series of studies by Laviolette and colleagues (2002, 2004) have shown that microinjections of nicotine into the ventral tegmental area (VTA) of the brain evoke a complex pattern of responses, which depend upon the dose used. Their studies suggest that stimulation of the DA neurones that project from the VTA can result in an aversive response to nicotine. Notwithstanding these observations, the evidence that selective lesions of DA projections to the nucleus accumbens attenuate responding for amphetamine, cocaine and nicotine is robust and reproducible and theories for the neurobiological mechanisms that mediate the reinforcing properties of these drugs must be reconciled with this fact. The studies reported by Laviolette and colleagues employed a conditioned place aversion paradigm in which the drug

was given non-contingently into the VTA. These workers acknowledged that the self-administration paradigm, employed by many groups to investigate the reinforcing properties of nicotine, may measure a behaviourally distinct phenomenon and that their studies did not exclude the possibility that mesolimbic DA neurones may play a pivotal role in this process (Laviolette and van der Krooy 2004).

2.1 The Role of the Dopamine Projections to Accumbal Shell and Core in Nicotine Dependence

The studies outlined in the previous section have highlighted the evidence that mesolimbic DA pathways in the brain play an important role in mediating the reinforcing properties of nicotine. Human smokers are exposed to nicotine on a chronic basis, which is very difficult to model adequately in animal studies. It has, nevertheless, become clear that the mesoaccumbens DA responses to nicotine are changed by repeated or chronic exposure to the drug and that the nature and extent of this plasticity may be important to our understanding of the neurobiological mechanisms underlying nicotine dependence. Similar to other drugs of dependence, acute intravenous injections of nicotine to drug-naïve animals stimulate DA overflow in the accumbal shell (as measured using microdialysis) but have little or no effect on DA overflow in the accumbal core (Pontieri et al. 1996). A similar regionally selective response is observed in rats given nicotine subcutaneously (Cadoni and Di Chiara 2000; Iyaniwura et al. 2001). Benwell and Balfour (1992) reported that daily subcutaneous injections of nicotine, given non-contingently by the experimenter, causes sensitisation of its effects on DA overflow in the nucleus accumbens. Subsequent studies have demonstrated that this effect is specific to the core subdivision of the structure (Cadoni and Di Chiara 2000; Iyaniwura et al. 2001; Fig. 1). By contrast, a more recent study by Lecca et al. and colleagues (2006) suggests that if the drug is self-administered, being contingent upon a lever-pressing response, the effects of the drug on DA overflow in the medial shell subdivision of the accumbens is enhanced in animals that have acquired the response over a 3-week period (Fig. 2). Although not emphasized by the authors, the data reported in Fig. 2 suggest that 3 weeks of contingent nicotine also increases DA overflow in the accumbal core. The results of experiments with other drugs of abuse, such as cocaine, have shown that the self-administration of these drugs is associated with a larger increase in DA overflow in the nucleus accumbens than in the accumbens of yoked animals given the same injections non-contingently (Hemby et al. 1997). Subsequent studies suggest that this sensitisation is mediated predominantly by the DA neurones that project to the accumbal shell (Lecca et al. 2006). Thus, it seems clear that the effects of chronic administration of nicotine, and other drugs of abuse, on DA overflow in the nucleus accumbens depend upon whether or not delivery of the drug is under the control of the animal and is contingent upon a specific learned response.

The neurobiological mechanisms that mediate the sensitised response to nicotine remain to be established. Mansvelder and colleagues (2000, 2002) have suggested

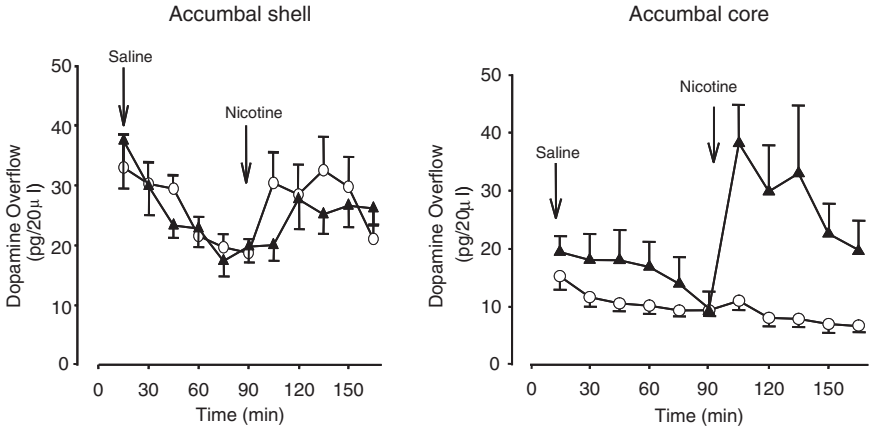


Fig. 1 Effects of acute and repeated non-contingent nicotine on dopamine overflow in the accumbal shell and core. The graphs demonstrate the effects of nicotine (0.4 mg kg^{-1} sc) injection on dopamine (DA) overflow in the accumbal shell (*left*) and core (*right*) when administered to drug-naïve animals (*open circles*) and animals pretreated with seven daily injections of nicotine prior to the test day (*solid triangles*). DA overflow was measured in conscious freely moving animals using microdialysis. Data represent means ± SEM. Nicotine exerted a significant ($P < 0.05$) effect on DA overflow in the accumbal shell of both drug-naïve and nicotine-pretreated animals. Pretreatment with nicotine did not significantly alter the response to nicotine injection in this subdivision of the accumbens. A nicotine challenge to drug-naïve animals had no significant effects on DA overflow in the accumbal core, but evoked a significant increase in DA overflow ($P < 0.01$) in animals that had been pretreated with nicotine for 7 days prior to testing. Derived from Iyaniwura et al. (2001)

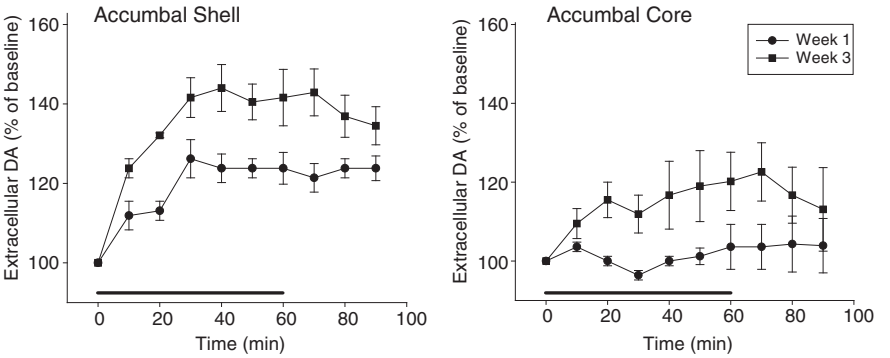


Fig. 2 Effects of self-administered nicotine on dopamine overflow in the accumbal shell and core. Rats were trained to self-administer nicotine (0.03 mg kg^{-1} per infusion). Microdialysis probes located in the accumbal shell and core were used to measure dopamine (DA) overflow during week 1 of training and in week 3 when the animals had acquired the response. Nicotine was available to the rats during the period indicated by the *bar*. The results are expressed as mean ± SEM. In the shell (*left*), nicotine stimulated DA overflow in both week 1 and week 3, the response in week 3 being higher ($P < 0.05$) than the response in week 1. In the core (*right*), DA overflow was only increased in week 3. Reproduced with permission from Lecca et al. (2006)

that the sustained effects of nicotine on DA overflow in the nucleus accumbens reflect a complex series of events within the VTA. The initial effects of nicotine are mediated by receptors composed of α4β2 subunits located on the DA neurones. The receptors mediate an initial depolarisation of the neurones. This response is followed rapidly by inhibition mediated by γ-aminobutyric acid (GABA)-secreting neurones located within the VTA or which project to the VTA from structures such as the accumbal shell. This inhibitory effect is mediated by presynaptic α4β2 nicotinic receptors located on GABA terminals, which facilitate GABA release. The α4β2 nicotinic receptors readily desensitise upon sustained exposure to nicotine, even if it is for a short time. On this point, Mansvelder and colleagues argue that the role of the α7 nicotinic receptors, located on glutamate terminals, becomes predominant because they remain active and require a higher concentration of nicotine before they desensitise. They suggest that continued stimulation of the receptors facilitates the release of glutamate and that this maintains the stimulation of the neurones and results in a prolonged release of DA in the nucleus accumbens. The hypothesis is supported by evidence that the effects of DA overflow in the accumbal shell depend, to some extent at least, upon the co-stimulation of NMDA receptors in the VTA (Schilström et al. 1998). There is evidence that the sensitised DA response observed in the accumbal core of nicotine-pretreated rats also depends on co-stimulation of NMDA receptors, although the anatomical location of these receptors has not been established (Shoaib et al. 1994; Balfour et al. 1996). It has been suggested that the increases in DA overflow evoked by nicotine in the accumbal shell and in the accumbal core of nicotine-sensitised rats reflect stimulation of NMDA receptors in the VTA, which enhances the proportion of mesolimbic DA neurones that exhibit burst firing (Balfour et al. 2000; Balfour 2004, 2006).

The mechanisms that mediate the regionally selective sensitisation of the DA responses to nicotine in the core and shell of the nucleus accumbens remain to be established. It seems reasonable to hypothesise that they reflect conditioned neurobiological responses to environmental stimuli associated with the delivery of nicotine. The VTA and accumbal shell receive significant glutamatergic projections from the ventral prefrontal cortex, whereas the accumbal core is innervated from the dorsal prefrontal cortex (Vanderschuren and Kalivas 2000). The nucleus accumbens is also innervated from the hippocampus. Stimulation of NMDA receptors in the dorsal hippocampus stimulates DA overflow in the accumbal core, whereas stimulation of NMDA receptors in the ventral hippocampus preferentially increases DA release in the accumbal shell (Peleg-Raibstein and Feldon 2006). Other studies suggest that neurones that project to the VTA from the pedunculopontine tegmentum also play a role in the development of sensitised locomotor responses to psychostimulant drugs and responding for nicotine in an intravenous self-administration paradigm (Alderson et al. 2003, 2006). Thus, DA overflow in the two subdivisions of the nucleus accumbens can be influenced preferentially or selectively by anatomical structures that have been implicated in the processing of conditioned stimuli.

The psychopharmacological significance of the changes in DA overflow that are evoked by chronic or repeated nicotine remains a matter for debate (Kelley and Berridge 2002). Results discussed in the previous section from the laboratories of

Rodd-Henricks and colleagues (2002) and Sellings and Clarke (2003) imply that the reinforcing properties of psychostimulant drugs of dependence depend on DA overflow in the shell subdivision of the accumbens. This conclusion is consistent with the neuroanatomical evidence that the accumbal shell forms part of an extended amygdala, a clearly limbic structure. In a series of articles, Di Chiara (1999, 2000a,b, 2002) has argued that increased DA release in the medial accumbal shell is to promote incentive or habit learning of behaviours that deliver rewards. As a result, acquisition of these behaviours is facilitated. Drugs of abuse, such as nicotine, exert their effects on this pathway through a pharmacological action and have the capacity to evoke increases in DA release that are unphysiologically large or prolonged. This can result, it is argued, in the development of compulsive drug-seeking behaviour (Di Chiara 1999, 2002).

Repeated injections of nicotine to experimental rats causes sensitisation of its effects on locomotor activity, sensitised locomotor stimulation being observed in rats pretreated with daily injections of the drug prior to the test day (Clarke and Kumar 1983; Clarke 1990). It was not surprising, therefore, that initially it was assumed that the sensitised DA responses to nicotine, observed in the accumbal core of animals pretreated with daily injections of the drug, mediated the sensitised locomotor responses observed in these animals (Benwell and Balfour 1992; Cadoni and Di Chiara 2000). However, the results of subsequent experiments cast doubt on this conclusion because they demonstrated that it was possible to dissociate completely the development and expression of the sensitised locomotor and accumbal DA responses to nicotine evoked by pretreatment with the drug. Thus, for example, Shoaib et al. (1994) and Balfour and colleagues (1996) reported that both the development and expression of the sensitised DA responses to nicotine, observed in the accumbal core, could be blocked by the co-administration of NMDA receptor antagonists, whereas these antagonists had no effects on the development or expression of the sensitised locomotor response to the drug. This observation led Balfour and colleagues (2000) to conclude that sensitisation of the DA response to nicotine was more likely to be involved in the development of nicotine dependence.

2.2 Mesolimbic Dopamine and Responding for Conditioned Reinforcers

Robinson and Berridge (1993, 2003) have long argued that the sensitisation observed in animals treated repeatedly with drugs of abuse plays a central role in the neurobiology underlying addiction. They have proposed that sensitisation of the mesolimbic DA projections to the nucleus accumbens is associated with the attribution of incentive salience to cues and stimuli associated with delivery of the drug, and that this sensitisation contributes to the mechanisms underpinning the "craving" for the drugs evoked by exposure to the stimuli. The hypothesis is supported by the observation that, in a second-order schedule of reinforcement, the non-contingent administration of a DA-releasing drug, D-amphetamine, enhances

responding for a conditioned reinforcer previously paired with a food reward (Taylor and Robbins 1984; Wyvell and Berridge 2000). Neither of the studies focused on the role of specific subdivisions of the accumbens. Other results, however, have shown that selective excitotoxin lesions of neurones in the accumbal core, but not the medial shell, attenuate the facilitation of responding for a food reward by the co-presentation of a conditioned stimulus (Hall et al. 2001). Furthermore, responding for a conditioned reinforcer paired with the delivery of cocaine is also attenuated by selective excitotoxin-evoked lesions of the accumbal core but not of the medial shell (Ito et al. 2004). These data, when considered together, provide strong support for the conclusion that neurones projecting to and from the core of the nucleus accumbens play a pivotal role in the neurobiological mechanisms that mediate the effects of stimuli associated with drug delivery on drug-seeking behaviour.

The role of the DAergic projections to the accumbal core in the responses to conditioned reinforcers or stimuli is less well established. An earlier self-administration study by Ito et al. (2000), in which the presentation of cocaine was associated with the presentation of a stimulus, showed that when cocaine was administered, extracellular DA levels in both the shell and core of the accumbens was increased. The results were anticipated because cocaine could be expected to inhibit DA reuptake in both subdivisions of the structure. Ito and colleagues then used a second-order schedule of reinforcement to show that lever pressing that was reinforced by the presentation of the conditioned stimulus alone had no significant effects of DA overflow in either the medial shell or the core of the accumbens. However, non-contingent presentation of the conditioned stimulus, a procedure commonly used to model relapse in animals in which responding for a drug reinforcer has been extinguished, evoked a regionally selective increase in DA overflow in the accumbal core. These data imply that responding *per se* for a reinforcer is not dependent upon increased DA overflow in either subdivision of the nucleus accumbens. Nevertheless, they suggest that Pavlovian drug-seeking behaviour is promoted by increased DA release in the accumbal core, a mechanism that may play an important role in relapse in abstinent individuals exposed to such stimuli (Ito et al. 2000).

Experiments of this type have yet to be performed with nicotine and it is only possible to speculate at this time upon the extent to which conditioned stimuli may also contribute to the mechanisms underlying nicotine dependence. However, there is considerable evidence to suggest that sensory stimuli play a central role in the addiction to nicotine and tobacco. Rose et al. and coworkers (1993) were amongst the first to report that sensory cues, present in tobacco smoke, are fundamentally important to the regulation of smoking behaviour and have a significant effect on the craving to smoke. More recent studies (Rose et al. 2000) have shown that these non-nicotinic sensory stimuli play a central role in the reinforcing effects of tobacco smoke and can satisfy a majority of the cravings to smoke. These stimuli seem to be particularly important in highly addicted smokers (Brauer et al. 2001).

In studies with experimental animals, the reinforcing properties of nicotine seem to be relatively weak and do not appear to be sufficiently powerful to explain the highly addictive nature of tobacco smoke (Donny et al. 2003; Balfour 2004). An early study by Goldberg and colleagues (1981) using squirrel monkeys showed that

the co-presentation of a visual stimulus significantly enhanced responding for intravenous nicotine. In more recent studies, in which rats have been trained to respond for nicotine, a conditioned stimulus is also commonly incorporated into the schedule to facilitate and enhance responding for the drug (e.g. Caggiula et al. 2001, 2002). In these studies, responding for nicotine was doubled by the co-presentation of the visual stimulus. These data suggest that the sensory stimulus plays a very important role in regulating nicotine-seeking behaviour. In experiments designed to explore the mechanisms that mediate the role of stimuli associated with exposure to nicotine, Caggiula and his colleagues have sought to compare the effects of nicotine, delivered contingently in response to a lever-pressing response, with the effects of non-contingent nicotine delivered using a yoked design in which the nicotine injections are controlled by the partner animal (Donny et al. 2003; Fig. 3). In agreement with previous studies, in animals in which the nicotine injections were contingent upon a lever-pressing response, the co-presentation of the compound visual stimulus

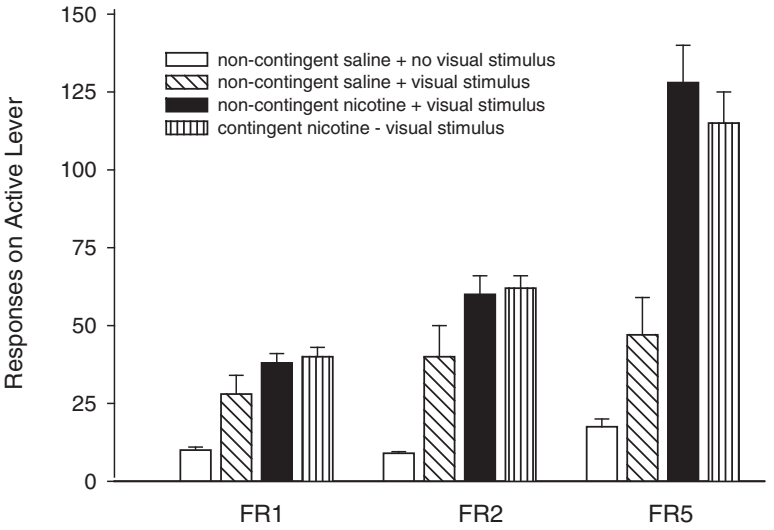


Fig. 3 Effects of nicotine on responding for a complex light stimulus. Rats were trained to respond for a complex light stimulus (stimulus light on over the active lever for 1 s; house lights off for 20 s) in an operant chamber. The 20 s period signalled by turning the house lights off, indicated a time-out period when responding on the active lever had no consequences. The rats received small iv infusions of nicotine, which were controlled by animals trained to respond for nicotine ($0.03\ mg\ kg^{-1}$ per infusion), which were yoked to the animals receiving non-contingent infusions of saline or nicotine. Data are expressed as mean ± SEM. Presentation of the light stimulus increased responding ($P < 0.05$) when compared with lever-pressing activity in the absence of the stimulus. This response was true for both saline- and nicotine-treated rats but was enhanced by non-contingent nicotine in a manner that depended upon the contingency. Significant increases in responding in the nicotine-treated rats were only significant ($P < 0.01$) when the contingency was increased to FR2 or FR5. Responding measured in rats given non-contingent nicotine injections was not significantly different to that measured in rats trained to associate the stimulus with self-administered injections of nicotine. Derived from Donny et al. (2003)

enhanced responding significantly, especially when the contingency was increased to FR5 (the rats were required to make five lever-pressing responses to receive the nicotine injection and the compound visual stimulus). However, in the yoked rats the rate of responding on the active lever for the compound visual stimulus was not significantly different from that observed in the partner rats able to control the delivery of nicotine. The results imply that nicotine has the ability to confer significant reinforcing properties on sensory stimuli that are otherwise weak reinforcers, and that this property of the drug may contribute significantly to the mechanisms underpinning (Palmatier et al. 2006; Balfour 2006).

The experimental design of the studies outlined above does not provide unequivocal support for the hypothesis that stimuli associated with the presentation of nicotine acquire the properties of a conditioned stimulus. A more recent study by Palmatier and colleagues (2007) has explored this possibility using a paradigm in which the conditioned reinforcing properties of the stimulus were transferred from one contingency to another. These experiments also confirmed that non-contingent nicotine can enhance responding for a stimulus that is clearly conditioned by prior association with the delivery of nicotine. Thus, the data seem to provide experimental support for the conclusions drawn by Rose and his colleagues, (1993, 2000) with regard to the role of sensory stimuli in the addiction to nicotine in human smokers.

2.3 The Putative Role of Extracellular Dopamine

The results summarised in the section above suggest that stimulation of DA projections to the two principal subdivisions of the nucleus accumbens facilitate and enhance responding for addictive drugs, but may not be entirely essential. Balfour (2004, 2006) has suggested that this conundrum might be solved if the increase in DA overflow in these areas of the brain, measured using microdialysis probes, reflects an increase in DA-mediated volume transmission within the accumbens. This hypothesis posits that DA released in this way stimulates extrasynaptic receptors bathed by transmitter in the extracellular space and that the primary consequence of increased stimulation of these receptors is to influence the probability that specific behaviours are exhibited. The hypothesis posits that increased DA overflow in the shell subdivision of the structure confers increased reinforcing, putatively hedonic, properties on behaviours associated with increased overflow. It is proposed that the psychophysiological purpose of the response is to facilitate acquisition of behaviours that result in rewarding outcomes (Fig. 4). However, drugs of dependence, particularly psychostimulants such as nicotine, enhance or sustain DA overflow from these neurones directly through their pharmacological effects on the DA neurones. Thus, the probability that an individual who takes a drug such as nicotine will repeat the behaviour (e.g. lever-pressing in rats/smoking in humans) which results in delivery of the drug is significantly enhanced.

The hypothesis predicts that increased DA overflow in the accumbal core plays a similar complementary role. It predicts that increased extracellular DA in this subdivision of the accumbens enhances the probability that animals will exhibit

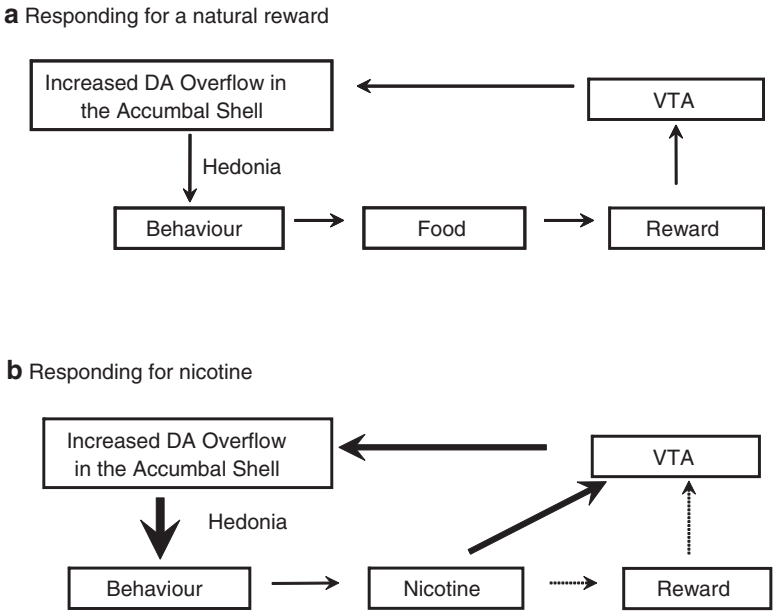


Fig. 4 Role of extracellular dopamine in responding for natural rewards (**a**) and for nicotine (**b**). This figure illustrates the way increased extracellular DA in the shell subdivision of the nucleus accumbens is postulated to increase the "pleasure" associated with behaviours that generate rewards. It is proposed that the behavioural role of the process is to facilitate the acquisition of behaviours that result in reward. The hypothesis proposes that the powerful reinforcing properties of drugs of dependence, such as nicotine, reflect their ability to act directly on the dopamine neurones that project to this subdivision of the accumbens. Reproduced with permission from Balfour (2006)

drug-seeking behaviour when presented with stimuli or cues associated with reinforcement (Balfour 2004, 2006). In abstinent individuals, this increases the probability of relapse. In individuals for whom the drug is also made available, the simultaneous increase in DA overflow in the shell and core subdivisions of the accumbens is predicted to drive the powerful craving for the drug that characterises dependence.

3 The Role of the Dorsal Striatum

There is evidence that nicotine also stimulates the DA projections to the dorsal striatum (Benwell and Balfour 1997; Quick 2004). To date, relatively few studies have sought to directly link this response to the drug with behavioural changes associated with dependence. Nevertheless, a number of studies have implicated these projections in the responses to rewarding stimuli, particularly in expectation of reward (Schultz 2006). A failure to deliver an anticipated reinforcer results in transient reduction in the activity of these neurones. Rice and Cragg (2004) have reported that

nicotine amplifies reward-related signals in the striatum. Thus, there is at least circumstantial evidence that the DA projections to the dorsal striatum are also likely to contribute to the neurobiology underlying nicotine dependence. Although it has not yet been explored in animals trained to respond for nicotine, studies in animals trained to respond for other psychostimulant drugs in a second-order schedule of reinforcement have shown that the presentation of a conditioned stimulus, which is contingent upon the animals making a response, is associated with increased DA overflow in the dorsolateral striatum (Ito et al. 2002). Furthermore, a recent study has shown that an increase in DA release in the dorsal striatum is not associated with an increased craving for cocaine unless coupled with cocaine-related cues (Volkow et al. 2008).

4 The Neurobiology Underlying Nicotine Withdrawal

Withdrawal of nicotine from experimental animals, following a period of chronic administration, evokes changes in behaviour that are thought to model components of the abstinence syndrome experienced by many smokers when they first quit their habit. These behavioural effects can be seen both as changes in spontaneous activity (Malin et al. 1992; Malin 2001) and measures of brain reward function (Epping-Jordan et al. 1998; Kenny and Markou 2001). In both models, early studies employed paradigms in which the drug was infused constantly from a subcutaneous osmotic minipump. The changes in spontaneous activity can be provoked by both the abrupt withdrawal of nicotine and the administration of a nicotinic receptor antagonist (Malin et al. 1992, 1994). The effects of abrupt nicotine withdrawal are reversed by the administration of a nicotine injection. These data imply that the abstinence syndrome, revealed using this model, reflects the sustained stimulation of neuronal nicotinic receptors. This is, perhaps, a surprising conclusion since it is to be expected that many neuronal nicotinic receptors are likely to be desensitised by sustained exposure to nicotine using the paradigms employed by Malin and colleagues to render their rats nicotine-dependent (e.g. Benwell et al. 1995; Pidoplichko et al. 1997). Studies employing nicotinic receptor antagonists and agonists that do not readily cross the blood–brain barrier suggest that many of the spontaneous changes in behaviour associated with nicotine withdrawal are mediated by changes mediated by nicotinic receptors in the periphery (Kenny and Markou 2001) although Malin and colleagues (1997) have reported that the abstinence syndrome in nicotine-pretreated rats is precipitated by the central, but not the peripheral, administration of the nicotinic antagonist, hexamethonium. It seems likely that both central and peripheral mechanisms contribute to the changes in spontaneous behaviour evoked by nicotine withdrawal.

The abstinence syndrome, evoked in animals by the withdrawal of nicotine, appears to be similar to that seen following opiate withdrawal (Malin et al. 1992). Furthermore, Malin and co-workers have reported that the abstinence syndrome can be provoked by the administration of the opiate antagonist, naxolone, to nicotine-treated rats (Malin et al. 1993). These results suggest that the abstinence syndrome

is also associated with excess stimulation of opiate receptors. This observation is difficult to reconcile with the evidence that another opioid antagonist, naltrexone, relieves the effects of withdrawal in abstinent smokers (Brauer et al. 1999; King and Meyer 2000).

One nicotine withdrawal effect that is clearly central in origin is the decrease in brain reward function measured using an intracranial self-stimulation (ICSS) paradigm (Epping-Jordan et al. 1998; Kenny and Markou 2001). This response is observed following either the abrupt withdrawal of nicotine, following a period of chronic infusion, or the administration of a nicotinic receptor antagonist to rats constantly infused with nicotine (Epping-Jordan et al. 1998; Watkins et al. 2000; Fig. 5). In rats in which the response is evoked by the abrupt withdrawal of nicotine, the symptoms are reversed by the administration of a nicotine injection (Epping-Jordan et al. 1998). A number of studies have sought to identify the neurobiological mechanisms that underpin this response to nicotine withdrawal. Hildebrand et al. and colleagues (1998) have shown that the precipitation of nicotine withdrawal evoked by the administration of the nicotinic receptor antagonist, mecamylamine, is associated with reduced DA overflow in the medial shell of the accumbens. These authors speculated that the reduction in DA overflow mediated the anhedonia associated with nicotine withdrawal. More recently, when discussing the possible mechanisms mediating the effects of nicotine withdrawal, Kenny and Markou (2001) speculated on the evidence that the abrupt withdrawal of drugs of dependence commonly results in decreased DA overflow in the nucleus accumbens and that this reduction in DA overflow mediates components of the aversive state associated with drug withdrawal. These authors concluded that the precise role of mesolimbic DA neurones in the symptoms of withdrawal, although attractive, must remain speculative.

Carboni and colleagues have reported that mecamylamine-precipitated withdrawal of nicotine increases DA overflow in the prefrontal cortex and have suggested that this increase in DA overflow may also contribute to the aversive consequences of abrupt nicotine withdrawal (Carboni et al. 2000). This response to nicotine withdrawal is not a universal finding since Hildebrand and colleagues failed to observe any changes in DA overflow in the prefrontal cortex following mecamylamine-precipitated withdrawal (Hildebrand et al. 1998). Thus, again, the putative role of these mesocortical projections in the behavioural responses to nicotine withdrawal remains unproven.

5 The Putative Role of Serotonergic Pathways in Nicotine Dependence

In a series of studies, Markou and her colleagues have sought to identify drugs that ameliorate the changes in brain reward function evoked by nicotine withdrawal (see Kenny and Markou 2001 for review). This review summarises the evidence that 5-hydroxytryptamine (5-HT) and, especially, 5-HT_{1A} receptors may play an important role in nicotine withdrawal, although the specific nature of the changes evoked

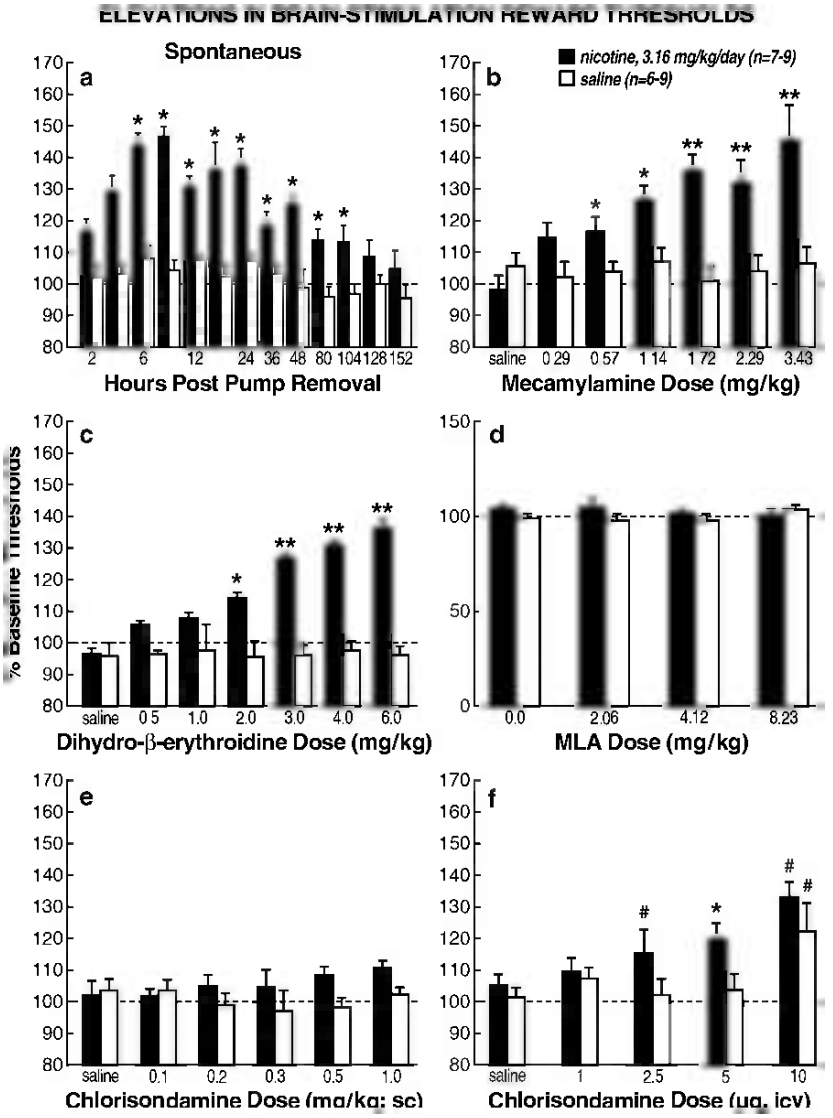


Fig. 5 Effects of nicotine withdrawal on brain reward thresholds. Nicotine withdrawal in rats is associated with elevations in brain reward thresholds. **a** Percentage of baseline reward thresholds in rats tested 2–152 h after removal of osmotic mini-pump delivering nicotine (3.16 mg kg^{-1} per day free base, 7 days). **b–f** Percentage of baseline thresholds in nicotine- and vehicle-treated rats after administration of: **b** mecamylamine (sc), **c** dihydro-β-erythrodine (sc), **d** methyllcaconitine (MLA) (sc), **e** chlorisondamine (sc), **f** chlorisondamine (icv). *Asterisks* indicate statistically significant differences between nicotine- and saline-treated rats (* $P < 0.05$, ** $P < 0.01$). *Hash symbols* indicate statistically significant difference in overall somatic withdrawal signs compared to 0.0 mg kg^{-1} chlorisondamine (# $P < 0.05$). All data are expressed as mean ± SEM overall somatic withdrawal signs at each time point or antagonist dose. Reproduced with permission from Kenny and Markou (2001). The data were derived from the studies of Epping-Jordan et al. (1998) and Watkins et al. (2000)

by nicotine withdrawal that can be attributed to changes in serotonergic function remain to be clarified. The conclusion is consistent with a series of results reported by Balfour and colleagues (Benwell and Balfour 1979, 1982; Benwell et al. 1990; Balfour and Ridley 2000), which suggest that nicotine decreases 5-HT release in the hippocampus of both experimental animals and smokers who inhale the drug in tobacco smoke and that chronic exposure to the drug evokes neuroadaptive changes in serotonergic function in this region of the brain. Furthermore, Seth and colleagues (2002) have summarised data suggesting that nicotine withdrawal may be associated with increased 5-HT release in the hippocampus. Benwell et al. (2000) observed that one of the neuroadaptive changes evoked in human brain by chronic smoking is a regionally selective increase in the density of 5-HT_{1A} receptors in the hippocampus. In an earlier study, Rasmussen et al. (1997) showed that the enhanced startle response evoked in rats by withdrawal of the drug could be ameliorated by the administration of a 5-HT_{1A} receptor antagonist. More recently, Harrison and colleagues (2001) showed that the co-administration of a 5-HT_{1A} receptor antagonist and a selective serotonin reuptake inhibitor could also ameliorate the effects of nicotine withdrawal on brain reward function, whereas this treatment had no effects on the somatic signs of withdrawal.

6 The Role of Metabotropic Glutamatergic Receptors in Behavioural Measures of Nicotine Dependence

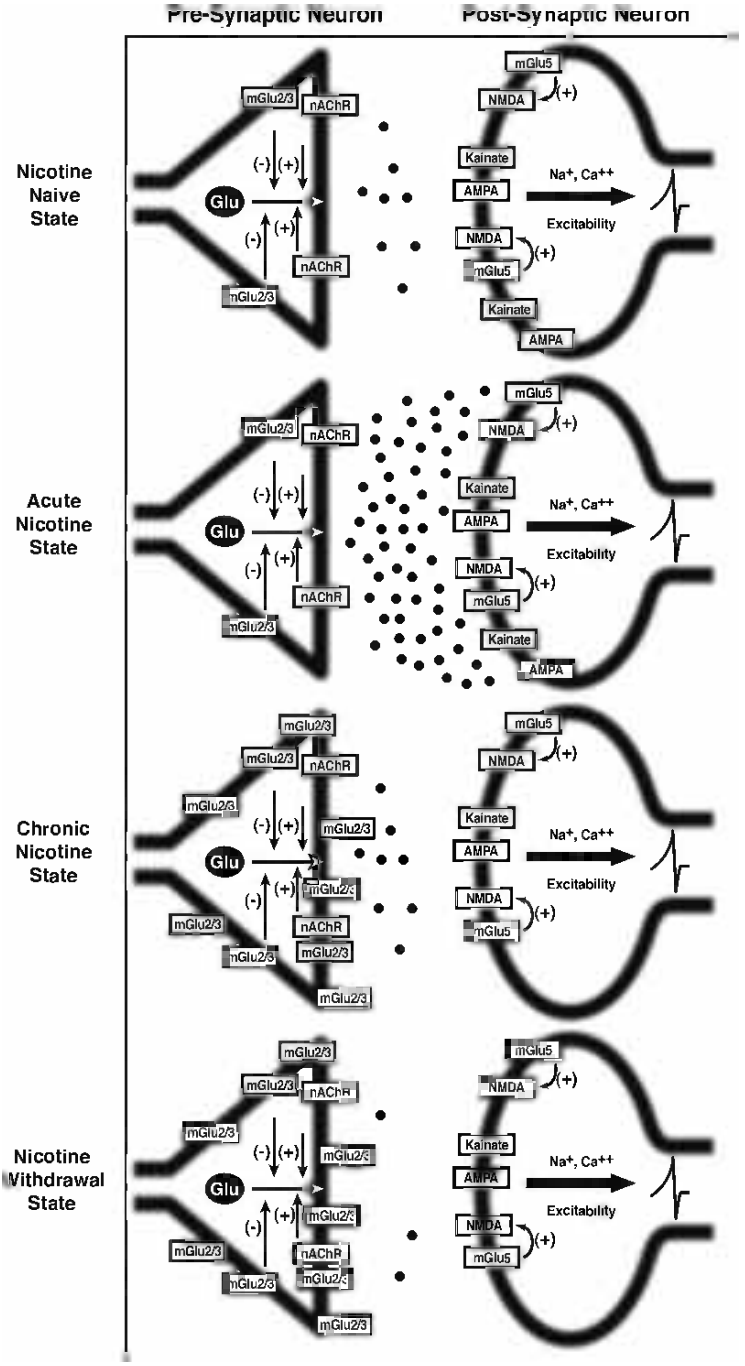
A series of experiments in Markou's laboratory have implicated metabotropic glutamate receptors in the mechanisms underpinning both the reinforcing properties of nicotine and the centrally mediated anhedonia associated with nicotine withdrawal. Thus, Kenny et al. (2003) reported that systemic injections of the mGluR2 receptor agonist, LY314582, decreased brain reward function (measured using the ICSS paradigm) in rats treated chronically with nicotine but not in rats treated with saline. That is to say, in nicotine-treated animals, evoked a change in behaviour that is characteristic of nicotine withdrawal. This effect was also observed in animals given bilateral microinjections of the antagonist into the VTA. The authors concluded that the data support the conclusion that the response to the drugs is mediated by their effects on mesoaccumbens DA neurones. A subsequent study (Liechti and Markou 2007) showed that the administration of the mGluR5 antagonist, (2-methyl-6-phenylethynyl)-pyridine (MPEP), decreased nicotine self-administration but exacerbated the effects of nicotine withdrawal on brain reward function. Additionally, this compound decreased brain reward function in saline-treated control rats. The response to MPEP was reversed by the co-administration of LY341495, a mGluR2/3 receptor antagonist. The mGlur2/3 receptors are thought to be inhibitory presynaptic autoreceptors located on glutamate terminals, whereas mGluR5 receptors are thought to be postsynaptic receptors, which modulate postsynaptic glutamatergic responses. Markou (2007) has rationalised the results employing mGluR agonists and antagonists by suggesting that, acutely, nicotine facilitates

the release of glutamate from glutamatergic terminals. This response to nicotine has rewarding or reinforcing properties, which depend upon the stimulation of post-synaptic glutamate receptors, putatively NMDA receptors, whose function is amplified by co-stimulation of mGluR5 receptors located on the same post-synaptic membranes (Fig. 6). Chronic administration of nicotine is hypothesised to enhance the inhibitory effects of the presynaptic mGluR2/3 receptors, thereby restoring glutamate release to "normal" in the presence of nicotine. However, when nicotine is withdrawn, the increased inhibitory control remains, reducing glutamate release. This results in reduced glutamatergic tone on mesoaccumbens DA neurones and decreased DA release in the nucleus accumbens.

7 The Role of Cannabinoid Receptors

Recent studies now suggest that endocannabinoid systems play an important role in mediating responding for drug abuse and for conditioned stimuli associated with drug delivery (Maldonado et al. 2006). A majority of the receptors in the brain that mediate the effects of endocannabinoids are CB1 receptors, the other principal cannabinoid receptor (CB2) being found predominantly in the periphery. The administration of CB1 receptor antagonists attenuates responding for many drugs of dependence of different pharmacological classes (e.g. morphine, heroin, ethanol, cocaine), as assessed using both self-administration and place preference paradigms. It seems likely that the ability to recruit endocannabinoid systems within the brain seems to be a property which is common to most, if not all, drugs of dependence (see Maldonado et al. 2006 for review). There is evidence that the CB1 antagonists can also attenuate both nicotine place preference (Le Foll and Goldberg 2004; Forget et al. 2005) and nicotine self-administration (Cohen et al. 2002; Shoaib 2008). Nicotine administration to transgenic mice lacking the CB1 receptor does not evoke the rewarding effect (measured using conditioned placed preference) observed in wild-type mice (Castane et al. 2002). Transgenic mice, however, do express a nicotine abstinence syndrome when it is precipitated with mecamylamine following a period of chronic treatment. Furthermore, the administration of CB1 receptor antagonists attenuates the persistent nicotine-seeking behaviour reinforced by the presentation of conditioned stimuli associated with delivery of nicotine (Cohen et al. 2005a), and the reinstatement of nicotine-seeking behaviour evoked by the non-contingent presentation of a priming dose of nicotine or a conditioned stimulus (Shoaib 2008). These observations suggest that CB1 antagonists diminish the reinforcing properties of both nicotine and cues associated with its delivery. They have resulted in these drugs being both proposed and explored as treatments for tobacco addiction (Cohen et al. 2005b).

The neurobiology underlying the effects of CB1 antagonists on nicotine-seeking behaviour remains to be established with certainty. However, there is convincing evidence that a number of the neurones that project to the VTA and nucleus accumbens express CB1 receptors (Maldonado et al. 2006; Cohen et al. 2005b). Importantly,


Pre-Synaptic Neuron
Post-Synaptic Neuron
Nicotine Naive State
Acute Nicotine State
Chronic Nicotine State
Nicotine Withdrawal State
Glu
mGlu2/3
nAChR
mGlu5
NMDA
Kainate
AMPA
(-)
(+)
Na+, Ca++
Excitability

the receptors are located on the terminals of GABA neurones that innervate DA-secreting neurones in the VTA. Furthermore, the glutamatergic projections that stimulate the GABA-secreting neurones in the nucleus accumbens, which project to the VTA also express these receptors. Thus, stimulation of these receptors by endocannabinoids or CB1 receptor agonists administered pharmacologically could be expected to stimulate mesolimbic DA neurones and increase DA overflow in the nucleus accumbens. The administration of Δ^9-tetrahydrocannabinol (Δ^9-THC), the active component of cannabis, or synthetic CB receptor agonists does indeed elicit a robust increase in DA overflow in the accumbal shell (Tanda et al. 1997).

Injections of CB1 antagonists block the effects of nicotine on DA overflow in the shell of the nucleus accumbens and it has been suggested that this effect of the drugs is pivotal to their ability to attenuate the reinforcing properties of nicotine (Cohen et al. 2002, 2005b). It is assumed, but has not yet been shown, that this response to the CB1 antagonists reflects attenuation of the inhibitory effects of endocannabinoids on receptors located on the GABA terminals that control DAergic activity within the VTA. Other studies have shown that rats can be trained to respond for microinjections of Δ^9-THC administered directly into the VTA, results which support the conclusion that the reinforcing properties of Δ^9-THC could be associated with disinhibition of mesoaccumbens DA neurones (Zangen et al. 2006). However, these authors reported that rats could also be trained to respond for microinjections of Δ^9-THC administered directly into the nucleus accumbens. Thus, it should not be assumed that there is necessarily a causative relationship between the effects of CB1 receptor antagonists on DA overflow in the accumbal shell and their effects on the reinforcing properties of nicotine and nicotine-associated cues. A more recent study has shown that bilateral microinjections of the CB1 antagonist, rimonabant, into the shell of the nucleus accumbens, the basolateral amygdala or the prelimbic cortex also attenuates cue-induced nicotine-seeking behaviour (Kodas et al. 2007). These results not only provide additional support for the conclusion that CB1 receptors

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Fig. 6 Schematic representation of a glutamate synapse in a nicotine-naïve state, during acute nicotine administration, during chronic nicotine exposure and during the early nicotine withdrawal phase. The presynaptic site contains inhibitory mGlu2/3 receptors and excitatory nAChRs that regulate the release of glutamate in the synapse. The postsynaptic site contains mGlu5, NMDA, kainate and AMPA receptors. The mGlu5 receptors interact positively with the NMDA receptors and increase NMDA receptor activity in a subtle way. *Top*: normal functioning of the system in a nicotine-naïve state. *Second diagram*: activation of nAchRs by nicotine administered through tobacco smoking increases glutamate release. Blockade of mGlu5 receptors prevents glutamate from having an action at these receptors, and thus blocks the reinforcing effects of nicotine. *Third diagram*: increased activity of inhibitory mGlu2/3 receptors (shown here as an increased number of receptors) and decreased activity of postsynaptic AMPA/kainate receptors (shown here as decreased number of receptors) that occur with chronic nicotine administration to restore glutamate release to pre-nicotine levels, reflecting the nicotine dependence state. When nicotine administration ceases (*bottom diagram*), the increased activity of presynaptic inhibitory mGlu2/3 receptors and decreased activity of postsynaptic AMPA/kainate receptors leads to decreased overall glutamate transmission, which presumably mediates the depression-like anhedonic state observed during nicotine withdrawal. Taken with permission from Markou (2007)

play a pivotal role in the maintenance of nicotine-seeking behaviour, but suggest that dependence does not depend solely on the recruitment of endocannabinoid systems located solely in the ventral tegmental area of the brain. Increased endocannabinoid activity in other cortico-limbic structures implicated in the neurobiology of dependence also seem to be involved in the expression of nicotine-seeking behaviour, at least as it is evoked by exposure to conditioned stimuli.

8 Conclusions

Experimental studies in the laboratory have shown that nicotine shares many of the pharmacological and behavioural properties that are exhibited by other drugs of dependence, particularly those with psychostimulant properties. Significantly, nicotine can serve as a reinforcer in a self-administration paradigm and this effect seems to depend critically on its ability to stimulate the mesolimbic DA neurones that project to the nucleus accumbens from the VTA. It seems reasonable to conclude, therefore, that these properties account for its role in the addiction to tobacco. However, whereas many smokers appear to become highly addicted to tobacco smoke, the addictive potential of nicotine, as assessed using laboratory models, seems to be relatively weak, in rodent models at least, and not to account fully for powerful addiction to tobacco. This review has considered several reasons why this should be true, including the fact that tobacco smoke contains constituents that may enhance the addictive potential of nicotine. The studies of Le Foll et al. (2007) also suggest that higher primates may be more sensitive to the reinforcing properties of nicotine than rodents and may model components of nicotine reinforcement that are more closely allied to those experienced by humans. However, this review has sought to develop hypotheses that explain how a drug with relatively weak intrinsic addictive properties can, nevertheless, play a pivotal role in the addiction to tobacco smoke. The effects of nicotine on neuronal pathways in the brain are complex because they are mediated by different subtypes of the nicotinic receptor and because sustained exposure to the drug often results in desensitisation of many of these receptors. This includes receptors located on mesolimbic DA neurones, which are thought to play a critical role in the reinforcing properties of nicotine. Behavioural studies suggest that sensory stimuli, paired with delivery of the drug, also play a pivotal role in nicotine-seeking behaviour in both experimental animals and smokers. Current hypotheses suggest that the salience of these stimuli may be associated with the prolonged increases in extracellular DA in the nucleus accumbens evoked when the drug is administered to an abstinent individual. The mechanisms responsible for this sustained response to nicotine are complex and not fully understood. However, contemporary studies suggest that glutamatergic and serotonergic systems may play important roles in this response to the drug. These observations have led Balfour (2006) to conclude that the tobacco smoking habit may best be described in terms of a second-order schedule in which smoking behaviour is largely reinforced by conditioned stimuli associated with inhaling tobacco smoke and the reinforcing

properties of nicotine itself are experienced only infrequently. If this hypothesis is correct, it provides an explanation for the relative lack of efficacy of "simple" treatments for tobacco dependence, such as nicotine replacement therapy, which do not address the important role of conditioned stimuli in the addiction to tobacco.

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Molecular Genetics of Nicotine Metabolism

Jill C. Mwenifumbo and Rachel F. Tyndale

Contents

Abstract The molecular genetics of nicotine metabolism involves multiple polymorphic catalytic enzymes. Variation in metabolic pathways results in nicotine disposition kinetics that differ between individuals and ethnic groups. Twin studies indicate that a large part of this variance is genetic in origin, although environmental influences also contribute. The primary aim of this chapter is to review the current knowledge regarding the genetic variability in the enzymes

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that metabolize nicotine in humans. The focus is on describing the genetic polymorphisms that exist in cytochromes P450 (CYPs), aldehyde oxidase 1 (AOX1), UDP-glucuronosyltransferases (UGTs), and flavin-containing monooxygenase 3 (FMO3). Genetic studies have demonstrated that polymorphisms in CYP2A6, the primary enzyme responsible for nicotine breakdown, make a sizable contribution to the wide range of nicotine metabolic capacity observed in humans. Thus, special attention will be given to CYP2A6, because slower nicotine metabolism requires less frequent self-administration, and accordingly influences smoking behaviors. In addition, the molecular genetics of nicotine metabolism in nonhuman primates, mice, and rats will be reviewed briefly.

Abbreviations

CYPs	Cytochromes P450
CYP2A6	Cytochrome P450 2A6
CYP2A13	Cytochrome P450 2A13
CYP2B6	Cytochrome P450 2B6
AOX1	Aldehyde oxidase 1
UGTs	UDP-glucuronosyltransferases
FMO3	Flavin-containing monooxygenase 3
SNPs	Single nucleotide polymorphisms
CAR	Constitutive androstane receptor
PGC-1α	Peroxisome proliferators-activated receptor-γ coactivor-1α
PXR	Pregnane X receptor
HNF-4α	Hepatocyte nuclear factor-4α
NNK	4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone
AUC	Area under the concentration time curve
UTR	Untranslated region
TMAU	Trimethlyaminuria
TMA	Trimethlyamine
cDNA	Coding deoxynucleotide acid
mRNA	Messenger ribonucleic acid
UDP	Uridine diphosphate

1 Introduction

The metabolism of nicotine involves multiple polymorphic catalytic enzymes. The primary aim of this chapter is to review current knowledge regarding the genetic variability of enzymes that metabolize nicotine in humans. Specific focus will be placed on the description of genetic polymorphisms that exist in cytochromes P450 (CYPs), aldehyde oxidase 1 (AOX1), UDP-glucuronosyltransferases (UGTs), and

flavin-containing monooxygenase 3 (FMO3). Variation in **CYP2A6** makes the most sizable contribution to the wide range of nicotine metabolic capacity observed in humans. Therefore, mention will be made of the regulators, inducers, and inhibitors that affect CYP2A6 level and activity. In addition, the molecular genetics of nicotine metabolism in nonhuman primates, mice, and rats will be described briefly.

2 Humans

2.1 Human Nicotine Metabolism

In general, 70–80% of nicotine undergoes sequential oxidation reactions, mediated by CYPs (Messina et al. 1997; Nakajima et al. 1996a) and AOX1 (Brandange and Lindblom 1979), to form cotinine (Benowitz et al. 1994). Cotinine is further oxidized by CYPs to *trans*-3′-hydroxycotinine (Nakajima et al. 1996b). Nicotine, cotinine, and *trans*-3′-hydroxycotinine are all glucuronidated by UGTs (Kaivosaari et al. 2007; Yamanaka et al. 2005a). Together, nicotine and the above-mentioned metabolites constitute most of a nicotine dose recovered in urine. Specifically, free and glucuronidated *trans*-3′-hydoxycotinine average 43%, free and glucuronidated cotinine average 26%, and free and glucuronidated nicotine average 14% of a nicotine dose recovered in urine (Benowitz et al. 1994; Byrd et al. 1992). Nicotine *N*′-oxide is a minor urinary metabolite at approximately 4–7% (Benowitz et al. 1994; Byrd et al. 1992), and its formation is catalyzed by FMO3 (Cashman et al. 1992). Minor metabolites, nornicotine at 0.7% (Benowitz et al. 1994) and norcotinine at 2% (Byrd et al. 1992), are likely produced by CYP2A6, CYP2A13, and CYP2B6 via demethylation (Murphy et al. 2005; Yamanaka et al. 2005b).

2.2 The Genetics of Human Nicotine Metabolism

In humans, there is considerable interindividual variation in the rate, extent, and pattern of nicotine metabolism in vitro (Messina et al. 1997) and in vivo (Benowitz et al. 1994). Such variation can arise from both genetic and environmental factors. Twin studies allow an estimate of the relative contribution of heritability (i.e., contribution of genes) and environmental influences to a particular phenotype. An investigation in twins of primarily Caucasian ethnicity found that 59% of the variance in nicotine clearance can be attributed to genetic influences (Swan et al. 2005).

2.3 Cytochromes P450: Genetic Polymorphisms Associated with Nicotine Metabolism

The main route of nicotine metabolism is through hepatic oxidation. In vitro, nicotine is oxidized by CYPs at the 5′ carbon (C-oxidation) of the pyrrolidine ring,

creating the unstable nicotine-$\Delta 1'(5')$-iminium ion intermediate, which is then rapidly converted to cotinine by AOX1, most likely in a non-rate-limiting manner (Brandange and Lindblom 1979). Of the hepatic CYPs, cDNA-expressed CYP2A6, has the greatest capacity to produce cotinine, followed by CYP2B6 and CYP2D6 (Yamazaki et al. 1999). In human liver microsomes, nicotine C-oxidation activity correlates with levels of immunoreactive CYP2A6 protein, and these protein levels can explain up to 88% of the variability in nicotine C-oxidation activity (Messina et al. 1997). Pharmacological and immunological inhibition experiments conclusively determined that CYP2A6 is the primary enzyme mediating cotinine formation in human liver microsomes (Messina et al. 1997; Nakajima et al. 1996a). Cotinine is further metabolized to *trans*-3′-hydroxycotinine in a reaction thought to be exclusively mediated by CYP2A6 in human liver microsomes (Nakajima et al. 1996b). A common finding of in vitro studies is the large interindividual variability in CYP2A6 protein levels, nicotine C-oxidation, and cotinine hydroxylation activities.

2.4 Cytochrome P450 2A Gene Cluster and CYP2A6 Polymorphisms

The *CYP2A6* gene is located in the *CYP2ABFGST* gene cluster on chromosome 19q13.2 (Hoffman et al. 2001); it spans approximately 6 kb, and consists of nine exons encoding 494 amino acids. *CYP2A6* is genetically variable; it can be deleted or duplicated, and can contain gene conversions, nucleotide deletions, nucleotide insertions, and single nucleotide polymorphisms (SNPs). For a current list of polymorphisms, please consult the *CYP2A6* allele nomenclature website (http://www.cypalleles.ki.se/cyp2a6.htm). Currently, 31 numbered (i.e., *CYP2A6*1–*31*) and two duplication (i.e., *CYP2A6*1X2A* and **1X2B*) *CYP2A6* alleles have been described. These include a wild-type allele (*CYP2A6*1*), an allele with a SNP in the noncoding promoter region (*CYP2A6*9*), 22 alleles with at least one nonsynonymous SNP (which results in an amino acid change) (*CYP2A6*2, *5, *6, *7, *8, *10, *11, *13, *14, *15*, **16, *17, *18, *19, *21*, **22, *23, *24, *25, *26, *28,* and **31*), frameshift alleles (which results in truncated proteins) (*CYP2A6*20* and **27*), gene conversion alleles (*CYP2A6*3* and **12*), deletion alleles (*CYP2A6*4*), and duplication alleles (*CYP2A6*1X2*). *CYP2A6*2, *4, *7, *10*, **17, *20, *23, *24, *26,* and **28* dramatically reduce CYP2A6 activity towards nicotine in vivo (Benowitz et al. 2001; Fukami et al. 2005a, 2007; Ho et al. 2008; Mwenifumbo et al. 2008; Xu et al. 2002). *CYP2A6*5, *6, *11, *19,* and **27* are predicted to dramatically reduce CYP2A6 activity toward nicotine in vivo, because their cDNA-expressed proteins have decreased or no enzyme activity (Daigo et al. 2002; Fukami et al. 2005b; Kitagawa et al. 2001; Mwenifumbo et al. 2008; Oscarson et al. 1999). *CYP2A6*9*, **12*, and **25* are associated with modestly reduced nicotine metabolism either in vitro or in vivo (Mwenifumbo et al. 2008; Oscarson et al. 2002; Yoshida et al. 2003). *CYP2A6*13*, **15*, and **22* are predicted to have reduced nicotine metabolism in vivo, due to specific SNPs that they contain

(Haberl et al. 2005; Kiyotani et al. 2002). *CYP2A6*8, *14, *16,* and **18* have not been associated with lower nicotine metabolism in vivo (Fukami et al. 2005b; Nakajima et al. 2006; Xu et al. 2002). *CYP2A6*1B* is associated with increased mRNA, protein level, and activity in vitro (Wang et al. 2006), and with moderately increased nicotine metabolism in vivo (Mwenifumbo et al. 2007a) in some, but not all, populations (Mwenifumbo et al. 2008). There is evidence that the two types of duplication alleles (*CYP2A6*1X2A* and **1X2B*) may result in increased nicotine metabolism (Fukami et al. 2007; Rao et al. 2000). Several additional SNPs (not yet assigned to alleles) remain to be characterized with respect to their haplotype, frequency, and functional impact on enzyme activity.

2.5 Cytochrome P450 2A6 Polymorphisms and Nicotine Metabolism

CYP2A6 decreased- or loss-of-function alleles are associated with impaired CYP2A6 activity and nicotine C-oxidation in vivo (Benowitz et al. 2006a; Malaiyandi et al. 2006; Mwenifumbo et al. 2008; Nakajima et al. 2006; Peamkrasatam et al. 2006), and their frequencies vary across different ethnic groups (Fig. 1). *CYP2A6* genotype has a substantive impact on in vivo nicotine C-oxidation capacity, and this has been demonstrated using different methods of nicotine administration, kinetic assessment measures, and ethnic groups. Specifically, *CYP2A6* genotype is associated with total clearance, nonrenal clearance, clearance of nicotine to cotinine, half-life, fractional conversion of nicotine to cotinine, and *trans*-3′-hydroxycotinine/cotinine (3HC/COT), a proxy measure of CYP2A6 activity (Benowitz et al. 2006a; Dempsey et al. 2004). Pharmacokinetic data acquired during a nicotine replacement clinical trial also showed that having at least one loss-of-function *CYP2A6* allele resulted in 50% lower CYP2A6 activity at baseline, and 44% higher steady-state plasma levels of nicotine achieved from the transdermal patch (Malaiyandi et al. 2006). The combined frequency of currently characterized *CYP2A6* decreased- or loss-of-function alleles is 9, 22, 43, and 51% in Caucasian, African American, Korean, and Japanese populations, respectively (Nakajima et al. 2006). Consistent with other studies, within each ethnic group, individual variant *CYP2A6* genotypes tended to have lower metabolic activity (Nakajima et al. 2006). Notably, a common observation in pharmacogenetic/pharmacokinetic studies is the considerable variability that exists in the *CYP2A6* 'wild-type' group (i.e., those without the assessed variant alleles) (Fig. 2). This may be due to unidentified *CYP2A6* polymorphisms or polymorphic proteins involved in the transcription or translation of the gene. Additionally, there are several other genetic and environmental contributors to nicotine C-oxidation variability, such as gender (Benowitz et al. 2006b; Mwenifumbo et al. 2007b) and smoking status (Benowitz and Jacob 2000).

The endogenous function of CYP2A6 is unknown; however, the enzyme seems to be nonessential, as evidenced by the gene deletion allele (i.e., *CYP2A6*4*) where

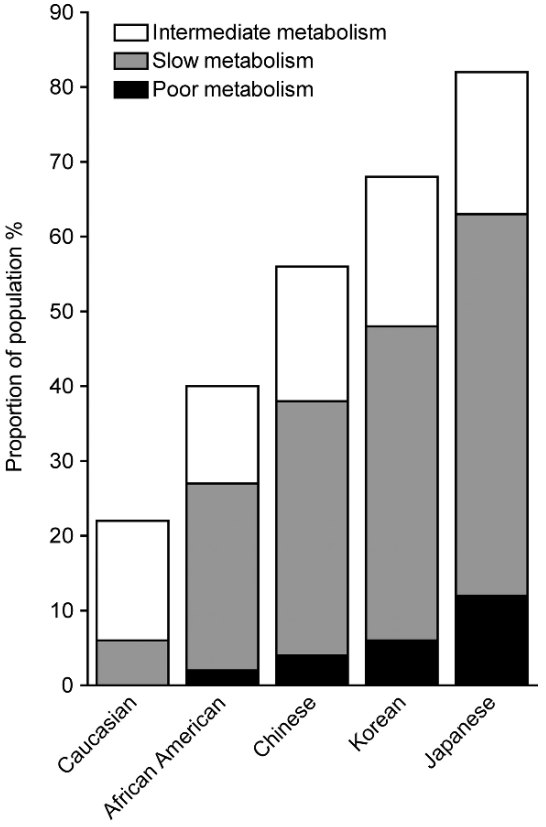

Fig. 1 Ethnicities vary in the proportion of persons with intermediate, slow, and poor nicotine metabolism, as predicted by *CYP2A6* genotype. Genotype frequencies were calculated based on the combined frequencies of *CYP2A6* predicted decrease-of-function alleles (D: *CYP2A6*9*, **12*, **13*, and **15*) and loss-of-function alleles (L: *CYP2A6*2, *4, *5, *6, *7, *10, *11, *17, *19*, and **20*) (Mwenifumbo et al. 2005; Nakajima et al. 2006; Schoedel et al. 2004). Grouping for the predicted nicotine metabolism groups were as follows: intermediate had one D allele, slow metabolism had one L allele or two D alleles, poor had the combination of one L and one D allele or two L alleles. Alleles *CYP2A6*23–*31* are not included in this figure

no functional protein is produced. Individuals homozygous for the gene deletion provide a classic illustration of the substantial impact *CYP2A6* genetics have on nicotine C-oxidation. Systemic levels of nicotine after oral administration are approximately four fold greater in individuals homozygous for the *CYP2A6* deletion (Fig. 3) (Xu et al. 2002). Furthermore, levels of urinary cotinine in smokers are dramatically (>85%) lower in those homozygous for the deletion (Yang et al. 2001). In the absence of CYP2A6 activity, the variability in other nicotine metabolic pathways is highlighted (Yamanaka et al. 2004).

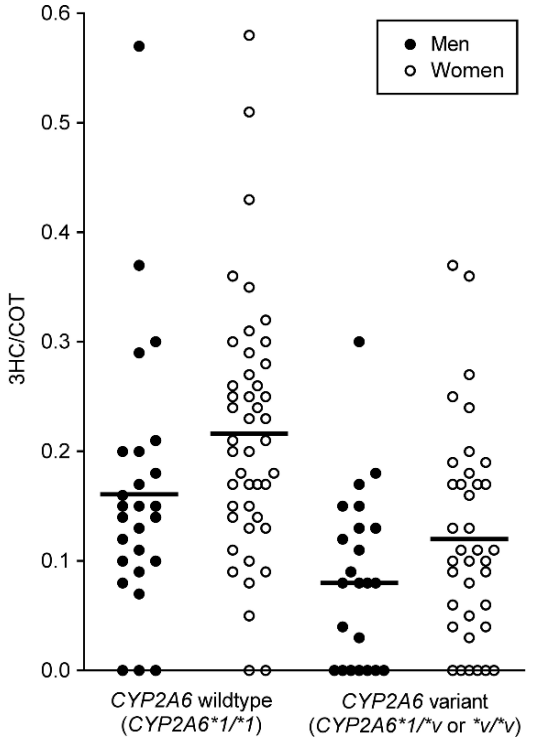

Fig. 2 CYP2A6 activity, as assessed by 3HC/COT, varies extensively in a nonsmoking population of black African descent after oral administration of 4 mg nicotine. Two major contributors to this variability are gender and *CYP2A6* genotype. In general, women have higher CYP2A6 activity compared to men, and persons without any assessed CYP2A6 genetics variant alleles (i.e., *CYP2A6*1/*1*, wild-type) have higher CYP2A6 activity compared to those with at least one variant allele (i.e., *CYP2A6*2, *4, *9, *12, *14, *15, *16, *17, *20*, and **21*, variant). Notably, there is extensive variability in the group genotyped as *CYP2A6*1/*1*, suggesting novel alleles. Adapted from Mwenifumbo et al. 2007b

2.6 Cytochrome P450 2A6 Genotype and Smoking

Nicotine is the primary compound in tobacco that establishes and maintains dependence (Henningfield et al. 1985), and smokers adapt their smoking behaviors to maintain preferred nicotine levels (Benowitz and Jacob 1985). Genetic variation in *CYP2A6* affects the pharmacokinetics of nicotine and smoking behaviors (reviewed in Malaiyandi et al. 2005). *CYP2A6* decreased- or loss-of-function variants have been associated with an altered risk of becoming nicotine-dependent (Audrain-McGovern et al. 2007; O'Loughlin et al. 2004), lower likelihood of being a smoker (Iwahashi et al. 2004; Schoedel et al. 2004), lower cigarette consumption (Malaiyandi et al. 2006; Minematsu et al. 2006; Schoedel et al. 2004), reduced inhalation (Strasser et al. 2007), and greater likelihood of cessation (Gu et al. 2000). However, these findings have not been uniformly confirmed (Munafo et al. 2004).

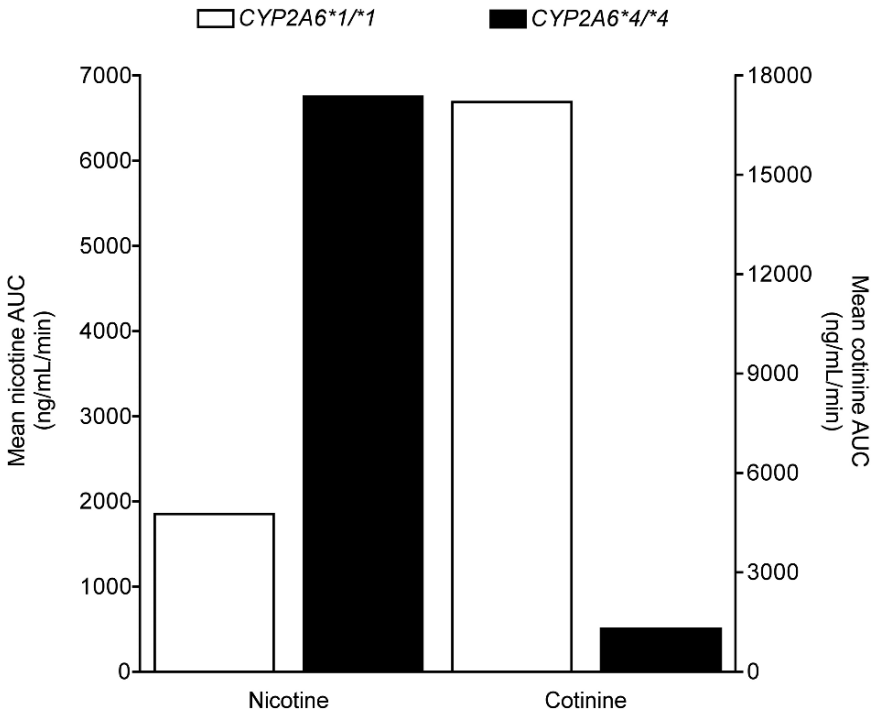

Fig. 3 Individuals missing both copies of the *CYP2A6* gene have substantially higher systemic exposure to orally administered nicotine and form very little cotinine. The *CYP2A6*4/*4* (homozygous deletion, $n = 3$) group has 365% higher systemic exposure (plasma AUC_{360} of nicotine) compared to the *CYP2A6*1/*1* (wild-type, $n = 5$) group, after oral administration of 4 mg nicotine. The homozygous deletion group forms only 9% of the cotinine of the homozygous wild-type group. Adapted from Xu et al. 2002

2.7 Cytochrome P450 2A6 Regulation

The amount of an enzyme expressed can be modulated by different molecular mechanisms. For example, inducers can result in an increase of gene transcription, mRNA stabilization, translational efficiency, and/or protein stabilization. CYP2A6 activity can be increased at the transcription and translation levels in response to a wide variety of drugs and other environmental molecules. Hepatic CYP2A6, as assessed at the level of mRNA, protein, or activity, may be induced by a wide variety of xenobiotics, such as phenobarbital (Meunier et al. 2000), rifampicin (Pichard-Garcia et al. 2000), dexamethasone (Meunier et al. 2000), pyrazole (Donato et al. 2000), the phytoestrogen biochanin A (Moon et al. 2007), cadmium (Satarug et al. 2004), and even dietary constituents such as broccoli (Hakooz and Hamdan 2007). However, upon exposure to inducers, the extent of induction tends to be modest, and varies between individuals (Parkinson et al. 2004). This interindividual variation may be partially due to genetic polymorphisms in the molecular machinery that

mediates the increased transcription or translation of *CYP2A6*. CYP2A6 induction has been linked to a number of polymorphic nuclear receptor proteins, including constitutive androstane receptor (CAR, gene *NR1I3*) (Lamba et al. 2005; Wortham et al. 2007), peroxisome proliferators-activated receptor-γ coactivator-1α (PGC-1α, gene *PPARGC1*) (Itoh et al. 2006; Kim et al. 2005), pregnane X receptor (PXR, gene *NR1I2*) (Itoh et al. 2006; Lim and Huang 2007), and hepatocyte nuclear factor-4α (HNF-4α, gene *HNF4A*) (Fukushima-Uesaka et al. 2006; Pitarque et al. 2005). Genetic and environmental variability modify xenoreceptor levels, and these levels have been associated with altered mRNA, protein, or activity of nicotine metabolizing enzymes such as CYP2A6 (Wortham et al. 2007), CYP2B6 (Chang et al. 2003), and some UGTs (Gardner-Stephen et al. 2004). This may contribute to the large interindividual variation in nicotine metabolic activity seen in persons with *CYP2A6* wild-type genotypes (Benowitz et al. 2006a; Nakajima et al. 2006) (Fig. 2).

Alternatively, xenobiotic exposure may inhibit or down-regulate CYP2A6. Nicotine (Schoedel et al. 2003), colfibric acid (Donato et al. 2000), and an hepatotrophic growth factor (i.e., augmenter of liver regeneration) (Thasler et al. 2006) result in lower levels of CYP2A mRNA, protein, and/or activity. Likewise, specific drugs and toxins can inhibit CYP2A6 enzymatic activity; these include α-naphthoflavone (Pelkonen et al. 1985), SKF 525A (Pelkonen et al. 1985), metyrapone (Draper et al. 1997; Pelkonen et al. 1985), aniline (Pelkonen et al. 1985), clotrimazole (Draper et al. 1997), diethyldithiocarbamate (Draper et al. 1997), ellipticine (Draper et al. 1997), ketoconazole (Draper et al. 1997), 8-methoxypsoralen (Zhang et al. 2001), 4-methylpyrazole (Draper et al. 1997), miconazole (Draper et al. 1997), *p*-nitrophenol (Draper et al. 1997), tranylcypromine (Draper et al. 1997), and tryptamine (Zhang et al. 2001).

2.8 Cytochrome P450 2A13

As discussed, persons homozygous for the *CYP2A6* gene deletion are capable of forming small amounts of cotinine from nicotine, probably via small contributions from other cytochromes P450 capable of nicotine C-oxidation. In humans, there are three complete *CYP2A* genes: *CYP2A6*, *CYP2A7,* and *CYP2A13* (Hoffman et al. 2001). cDNA-expressed CYP2A7 is enzymatically inactive (Yamano et al. 1990). Conversely, expressed CYP2A13 is very efficient in catalyzing nicotine C-oxidation and cotinine hydroxylation (Bao et al. 2005). However, CYP2A13 mRNA levels are highest in the human respiratory tract and very low in the liver (Su et al. 2000). Thus, CYP2A13 may not contribute substantively to systemic pharmacokinetic profiles of nicotine or cotinine, despite its high metabolic activity toward both substrates. It is noteworthy that expressed CYP2A13 is the most active human CYP in the metabolic activation of tobacco smoke precarcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) to its reactive intermediate, and thus may play an important role in tobacco-related lung cancer (Su et al. 2000).

In human lung tissue, CYP2A protein varies markedly between individual samples (Shimada et al. 1996), and the *CYP2A13* gene is polymorphic (Misra et al. 2007; Zhang et al. 2002). For a current list of polymorphisms, please consult the *CYP2A13* allele nomenclature website (http://www.cypalleles.ki.se/cyp2a13.htm). Currently, there are nine numbered alleles described (i.e., *CYP2A13*1–*9*). These include one wild-type allele, seven alleles with nonsynonymous SNPs, and one frameshift allele. Only *CYP2A13*2*, **3*,** 4*, and **7* are thought to result in a decrease- or loss-of-function (Wang et al. 2006b; Zhang et al. 2002), and there is marked variation in the frequency of these alleles across ethnic groups (Wang et al. 2006b).

The impact of *CYP2A13* polymorphisms on in vivo systemic nicotine metabolism and nicotine disposition kinetics has not been investigated, and is not predicted to be consequential in individuals with 'normal' CYP2A6 function. However, its expression in the lung, and thus its potential impact on tissue-specific metabolism, has made it an attractive candidate for investigating genotype and tobacco-specific lung cancer risk. Although still early, *CYP2A13* decrease- or loss-of-function polymorphisms have been associated with a decreased likelihood of lung cancer in a Chinese population (Wang et al. 2003a), but with an increased likelihood of lung cancer in a Caucasian population (Cauffiez et al. 2004).

2.9 Cytochrome P450 2B6

Originally named the phenobarbital-inducible cytochrome P450IIB, CYP2B6 is capable of nicotine C-oxidation, although it has only one-tenth the catalytic efficiency of CYP2A6 (Yamazaki et al. 1999). Based on human liver microsome studies, it has been suggested that, at higher nicotine concentrations, CYP2B6 may be involved in minor amounts of cotinine formation (Yamazaki et al. 1999).

CYP2B6 protein levels vary as much as 100-fold in human livers (Ekins et al. 1998; Lamba et al. 2003). Additionally, CYP2B6 varies between genders and across different ethnic groups. For example, liver samples from women had higher CYP2B6 mRNA, protein, and activity (Lamba et al. 2003). Also, CYP2B6 activity was as much as two fold greater in livers of Hispanics, compared to Caucasians and African Americans (Lamba et al. 2003). Some of this variability in protein and activity levels can be attributed to, and has been associated with, *CYP2B6* genetic polymorphisms. For a current list of polymorphisms please consult the *CYP2B6* allele nomenclature website (http://www.cypalleles.ki.se/cyp2b6.htm). Currently, there are 29 numbered alleles described (i.e., *CYP2B6*1–*29*), which include a wild-type allele, one allele with a SNP in the promoter region, 25 alleles with nonsynonymous SNPs, one frameshift allele, and one deletion allele. *CYP2B6* alleles have not been systematically characterized with respect to their effect on in vitro and/or in vivo nicotine metabolism activity.

CYP2B6 decrease- and loss-of-function alleles affect the rate and extent of drug metabolism, and hence may predict the clinical efficacy of drugs such as efavirez,

an antiretroviral used to treat persons with HIV/AIDS (Haas 2006). The impact of several *CYP2B6* polymorphisms on in vivo systemic nicotine metabolism and nicotine disposition kinetics has been informally investigated. Current evidence does not support a large contribution of CYP2B6 to peripheral nicotine metabolism. Specifically, a recent smoking cessation study in Caucasians found that *CYP2B6* decrease-of-function alleles did not alter nicotine plasma levels obtained from the transdermal patch, even among those with genetically reduced *CYP2A6* metabolism (Lee et al. 2007). Because CYP2B6 is highly inducible by various drugs and toxins, more so than CYP2A6 (Madan et al. 2003), it may contribute to nicotine C-oxidation under conditions of induction. For example, nonhuman primates chronically dosed with phenobarbital (a CYP inducer) excrete 42% less unchanged nicotine in urine (Seaton et al. 1991), and have 46% lower systemic nicotine exposure (AUC) (Lee et al. 2006a), both of which suggest increased oxidation activity. Moreover, with phenobarbital dosing, there is a substantial increase (6.5- to 56-fold) in CYP2B protein levels (Ohmori et al. 1993a; Schoedel et al. 2003), while CYP2A protein is only modestly induced (3.3-fold) in the liver (Ohmori et al. 1993b). Taken together, these findings suggest that, under circumstances of induction, some increase in CYP2B6-mediated nicotine metabolism may occur.

Smoking is associated with greater CYP2B6 levels in the human brain (Miksys et al. 2003). Nicotine itself is thought to mediate the induction of brain CYP2B, as has been demonstrated in rodents (Miksys et al. 2000) and nonhuman primates (Lee et al. 2006b). Thus, the level of this enzyme is influenced by environmental and genetic factors, and may have potential to modulate local brain nicotine psychopharmacology (Miksys and Tyndale 2004).

2.10 Aldehyde Oxidase 1 (AOX1)

Cytochromes P450 oxidize nicotine to nicotine-$\Delta 1'(5')$-iminium ion, which is subsequently oxidized to cotinine by cytosolic AOX1 (Brandange and Lindblom 1979). In smokers, 26–30% of absorbed nicotine is excreted in the urine as cotinine and its glucuronide (Benowitz et al. 1994; Byrd et al. 1992). AOX1 is a molybdenum flavoprotein involved in the metabolism of various endogenous and exogenous nitrogen-containing heterocyclic compounds (Kitamura et al. 2006). In human liver samples, AOX1 activity can vary two- to >50-fold, depending on the substrate tested (Al-Salmy 2001; Rodrigues 1994; Sugihara et al. 1997). In addition to interindividual variability, AOX1 activity measured in liver cytosol differed across two studies, one in Caucasians and the other in Japanese, suggesting interethnic variability in AOX1 (Rodrigues 1994; Sugihara et al. 1997). Gender, age (after 1-year of age), smoking status, and disease history do not seem to influence AOX1 activity in vitro or in vivo (Al-Salmy 2001; Tayama et al. 2007), and the enzyme does not appear to be easily induced (Kitamura et al. 2006). However, in vitro AOX1 can be substantially inhibited by many clinically used drugs, including raloxifene, estradiol, tamoxifen, and ketoconazole (Obach et al. 2004).

The *AOX1* gene maps to chromosome 2q33, and is approximately 86 kb long with 35 transcribed exons that encode a large and structurally complex protein (Garattini et al. 2003). Of the hundreds of SNPs reported in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), only eight result in amino acid changes, and none has been assessed in vitro or in vivo for their functional consequence. However, an in silico investigation reported that one nonsynonymous SNP would likely result in an inactive enzyme (Steinberg et al. 2007). Rare *AOX1* polymorphisms may be relevant to clinical disorders. For instance, AOX1 is implicated in the pathogenesis of amyotrophic lateral sclerosis (Berger et al. 1995). The potential impact of *AOX1* polymorphisms is currently unknown, but AOX1 may not greatly influence cotinine formation because the catalytic efficiency of AOX1 in metabolizing the nicotine-$\Delta 1'(5')$-iminium ion to cotinine is at least 25-fold greater than that of CYP2A6 in converting nicotine to the nicotine-$\Delta 1'(5')$-iminium ion (Obach 2004; Messina et al. 1997). Nonetheless, estradiol (an AOX1 inhibitor) is capable of inhibiting the conversion of the nicotine-$\Delta 1'(5')$-iminium ion to cotinine (Brandange and Lindblom 1979).

2.11 UDP-Glucuronosyltransferases (UGTs)

In smokers, 3–5% of absorbed nicotine is excreted in the urine as nicotine *N*-glucuronide (Benowitz et al. 1994; Byrd et al. 1992). However, in individuals homozygous for the *CYP2A6* gene deletion, and therefore deficient in nicotine C-oxidation activity, up to 46% of absorbed nicotine is excreted in the urine as nicotine *N*-glucuronide, indicating substrate metabolism rerouting (Yamanaka et al. 2004). Cotinine and *trans*-3′-hydroxycotinine also undergo glucuronidation, and typically 16–17% of absorbed nicotine is excreted in the urine as cotinine *N*-glucuronide, as well as an additional 8–9% as *trans*-3′-hydroxycotinine *O*-glucuronide (Benowitz et al. 1994; Byrd et al. 1992).

Glucuronidation is the process of enzymatically adding glucuronic acid to substrates; typically, the resulting glucuronide is more water-soluble and more readily excreted. The exact UDP-glucuronosyltransferases (UGTs) isoform/s that mediates nicotine and cotinine conjugation is unclear, but in vivo (Benowitz et al. 1994; Byrd et al. 1992) and in vitro (Nakajima et al. 2002) evidence suggests that the same UGT mediates both reactions. UGT2B10, a liver enzyme previously not known to exhibit considerable activity towards any compound (Jin et al. 1993), most likely mediates the glucuronidation of nicotine and cotinine (Kaivosaari et al. 2007). *Trans*-3′-hydroxycotinine undergoes O-glucuronidation, and this is thought to be mediated by UGT2B7 (Yamanaka et al. 2005a).

The *UGT2B* gene subfamily maps to chromosome 4q13, and discrete genes encode each UGT2B protein. The *UGT2B10* gene spans approximately 15 kb, consists of six exons, and encodes 528 amino acids. Similarly, the *UGT2B7* gene spans approximately 16 kb, consists of six exons, and encodes 529 amino

acids (Mackenzie et al. 2005). Numerous genetic variants in *UGT2B* genes have been described in recent years (Nagar and Remmel 2006). For a current list of alleles, please consult the UDP-glucuronosyltransferase alleles nomenclature home page (http://galien.pha.ulaval.ca). Of the numerous SNPs reported for the UGT2B10 gene in the NCBI dbSNP database, two SNPs result in amino acid changes (http://www.ncbi.nlm.nih.gov/SNP/). Chen and colleagues reported that the *UGT2B10*2* allele significantly reduced nicotine and cotinine N-glucuronidation, indicating that *UGT* genotype may play a role in nicotine elimination (Chen et al. 2007). A multitude of intronic and noncoding region polymorphisms have been reported for *UGT2B7*, including ten nonsynonymous and one frameshift SNP (http://www.ncbi.nlm.nih.gov/SNP/). Four numbered alleles are currently described (i.e., *UGT2B7*1–*4*) (http://galien.pha.ulaval.ca). Their impact on *trans*-3′-hydroxycotinine glucuronidation is presently unknown. Interestingly, the effect of some *UGT2B7* polymorphisms may be substrate-specific (Nagar and Remmel 2006).

In human liver microsomes, glucuronidation of nicotine, cotinine, and *trans*-3′-hydroxycotinine can vary up to 13- (Ghosheh and Hawes 2002), 17- (Ghosheh and Hawes 2002) and five fold (Yamanaka et al. 2005a), respectively. Moreover, there is interindividual and interethnic variability in the extent of nicotine *N*-glucuronide, cotinine *N*-glucuronide, and *trans*-3′-hydroxycotinine *O*-glucuronide formation (Benowitz et al. 1999; Byrd et al. 1992). For example, African American smokers excrete a lower percentage of nicotine and cotinine as their respective N-glucuronides, compared with Caucasians. This study provided evidence of a polymorphic distribution of N-glucuronidation, with slow metabolizers almost exclusively found in the African Americans (Benowitz et al. 1999).

2.12 Flavin-Containing Monooxygenase 3 (FMO3)

In smokers, approximately 4–7% of absorbed nicotine is excreted in the urine as nicotine *N′*-oxide (Benowitz et al. 1994; Byrd et al. 1992). However, in individuals homozygous for the CYP2A6 gene deletion, and therefore deficient in nicotine C-oxidation activity, up to 31% of absorbed nicotine is excreted as nicotine *N′*-oxide, indicating substrate metabolism rerouting (Yamanaka et al. 2004). Nicotine *N′*-oxide is formed by FMO3 (Cashman et al. 1992), which represents the major hepatic isoform in humans (Krueger and Williams 2005). Both nicotine N-oxidation (Cashman et al. 1992) and immunodetected FMO3 protein levels have been reported to vary in human liver microsomes by approximately six- and nine fold, respectively (Overby et al. 1997). Formation of nicotine *N′*-oxide in human liver microsomes does not appear to be dependent on gender, age, smoking history, or previous drug administration history (Cashman et al. 1992). Because FMO3 is not readily induced by xenobiotics (Krueger and Williams 2005), interindividual variation is likely due to genetic factors (Cashman et al. 2001). Variability in FMO3-mediated substrate metabolism may be due to interindividual, interethnic,

or route of administration differences. For example, urinary nicotine *N*-oxide varies approximately three fold with *ad libitum* smoking, 11-fold with nicotine intravenous infusion, and seven fold with the transdermal patch (Park et al. 1993).

The *FMO3* gene is located amidst a *FMO* gene cluster on chromosome 1q23-q25, and it spans 27 kb and contains one noncoding and eight coding exons (Hernandez et al. 2004). There are at least 40 *FMO3* alleles. For a current list of alleles, please consult the Allelic Variant Database website (http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3/). Allele frequencies differ between ethnic groups (Cashman et al. 2001). Loss-of-function variants of *FMO3* identified in individuals with extremely rare phenotypes may not be typical of genetic variation in the population in general (Cashman et al. 2001). For instance, trimethlyaminuria (TMAU) is an inherited disorder, colloquially known as fish odor syndrome. Functional FMO3 typically mediates the N-oxidation of malodorous trimethlyamine (TMA) to its nonodorous metabolite TMA *N′*-oxide (Al-Waiz et al. 1988). Loss-of-function *FMO3* alleles are associated with the TMAU phenotype (Treacy et al. 1998) as well as deficient nicotine N-oxidation (Ayesh et al. 1988). Common decrease-of-function *FMO3* alleles that have modest effects on N-oxidation activity, via slight modulation of protein levels and/or function, are more likely to contribute to general population variation in FMO3 (Cashman et al. 2001).

3 Nonhuman Primate Nicotine Metabolism

There are only a few studies of nonhuman primate nicotine metabolism. However, the mean half-life of nicotine in male stumped-tailed macaques (*Macaca arctoide*) is approximately 100 min (Seaton et al. 1991) and is approximately 200 min in male African green monkeys (*Cercopithecus aethiops*) (Lee et al. 2006a), and both these closely resemble the 95–227 min half-life in humans (Benowitz and Jacob 1994). The plasma and urinary profile of nicotine and its metabolites in the male stumped-tailed macaque are also comparable to those observed in humans, suggesting analogous nicotine metabolic pathways in nonhuman primates (Benowitz and Jacob 1994; Byrd et al. 1992; Seaton et al. 1991). Also similar to humans, nicotine C-oxidation predominates over N-oxidation in rhesus macaque (*Macaca mulatta*) hepatocytes (Poole and Urwin 1976), and approximately 80–90% of nicotine C-oxidation is mediated by a CYP2A6-like protein in African green monkey liver (Schoedel et al. 2003). CYP2A proteins are present in the livers of many primate species such as baboon (*Papio papio*) (Dalet-Beluche et al. 1992), cynomolgus monkey (*Macaca fascicularis*) (Pearce et al. 1992), marmoset (*Callithrix jacchus*) (Schulz et al. 2001), and squirrel monkey (*Saimiri Boliviensis*) (Ohmori et al. 1993b).

Recently, a comparison of the *CYP2* gene cluster was made among three primates species: humans, chimpanzees (*Pan troglodytes*), and rhesus macaques (Hoffman and Hu 2007). In great apes (i.e., humans and chimpanzees) the *CYP2ABFGST* clusters are very similar; the chimpanzee orthologs of human *CYP2* genes all have

92–99% of the nucleotide identity in common in the coding sequence (Hoffman and Hu 2007). Conversely, the organization and composition of the *CYP2ABFGST* cluster in the Old World monkey, rhesus macaque, is substantially different from humans (Hoffman and Hu 2007). However, even among the Old World monkeys, the *CYP2A* genes have >90% nucleotide identity in common in the coding sequence with their respective human counterparts (Hoffman and Hu 2007). Given the similarities between humans and nonhuman primates in urinary metabolite profile, in vivo nicotine kinetics, in vitro nicotine metabolic profile, in vitro nicotine C-oxidation activity, and the highly conserved *CYP2A* genes, some, but not all, nonhuman primates may serve as suitable animal models of human CYP2A6-mediated nicotine C-oxidation.

4 Mouse Nicotine Metabolism

There are several studies of nicotine metabolism and disposition kinetics in mice (*Mus musculus*) (Damaj et al. 2007; Petersen et al. 1984; Raunio et al. 2008; Siu and Tyndale 2007). Both the elimination of nicotine and the accumulation of metabolites are extremely rapid in mice, suggesting that metabolic clearance is the major route of elimination. The nicotine half-life averages approximately 6–9 min (Petersen et al. 1984; Siu and Tyndale 2007), which is substantially shorter than in rats at 54–66 min (Kyerematen et al. 1988), or in humans at 95–227 min (Benowitz and Jacob 1994). There are many similarities between human and mouse nicotine metabolism. First, mouse *Cyp2a5* has 84% nucleotides in common in the coding sequence compared to human *CYP2A6* (Wang et al. 2003b). Second, the majority (i.e., 70–100%) of in vitro hepatic nicotine C-oxidation is mediated by CYP2A5 (Raunio et al. 2008; Siu and Tyndale 2007). Third, plasma levels of cotinine predominate over nicotine N'-oxide in mice (Petersen et al. 1984). Fourth, of 3′-hydroxycotinine formation is almost exclusively mediated by CYP2A5 (Siu and Tyndale 2007). Fifth, as in humans, 3′-hydroxycotinine is the most abundant urinary metabolite (Raunio et al. 2008). Finally, methoxalen, an inhibitor of mouse CYP2A5 and human CYP2A6, decreases the metabolism of nicotine, and has been demonstrated to substantially increase the amount of nicotine excreted unchanged in mouse urine (Raunio et al. 2008) and increase the systemic exposure to nicotine in humans (Sellers et al. 2000).

There are slight interstrain differences in plasma nicotine kinetic parameters in mice (Petersen et al. 1984; Siu and Tyndale 2007), even though nicotine C-oxidation activity in liver microsomes is similar between strains (Siu and Tyndale 2007). However, the in vitro and in vivo disposition kinetics of cotinine and coumarin, and metabolism by CYP2A5, vary substantially between strains (Kaipainen et al. 1984; Siu and Tyndale 2007). These strain differences have been shown to be, at least in part, due to genetic differences in CYP2A5 protein regulation (Lang et al. 1989). For example, strains differ in the level of CYP2A5 protein (i.e., likely differences at the regulatory level), and thus in the enzyme activity (Lang et al. 1989; Siu et al. 2006). Structural differences in CYP2A5 (i.e., at the level of the amino acid) also exist

between mouse strains, as is true for human *CYP2A6*. Mouse *Cyp2a5* is genetically polymorphic, and there is one amino acid different between DBA/2 and C57BL/6 strains (Lindberg et al. 1992), which may cause a subtle change in substrate affinity between the CYP2A5 proteins of each strain (He et al. 2004).

Mouse CYP2A5 is a comparable isoform to human CYP2A6 (Raunio et al. 1988), because these isozymes are responsible for the majority of nicotine's C-oxidation (Raunio et al. 2008), cotinine's subsequent oxidation to 3′-hydroxycotinine (Siu and Tyndale 2007), and coumarin 7-hydroxylation (Kaipainen et al. 1984). Taken together, these findings suggest that mice may be a cost-effective animal model of human CYP2A6-mediated nicotine C-oxidation.

5 Rat Nicotine Metabolism

The principal measured metabolite in the plasma of rats (*Rattus norvegicus*) receiving nicotine is cotinine, but the most abundant urinary metabolites are unchanged nicotine and nicotine N'-oxide, not 3′-hydroxycotinine as in humans and mice (Kyerematen et al. 1988a). Moreover, similar amounts of cotinine and nicotine N'-oxide are produced in rat hepatocytes (Kyerematen et al. 1990). These data suggest that, unlike humans and mice, rat nicotine N-oxidization pathways play a larger role, and may even predominate over C-oxidation metabolic pathways. Rat hepatic CYP2A enzymes, CYP2A1 and CYP2A2, do not metabolize nicotine (Hammond et al. 1991). CYP2A3, an extrahepatic lung enzyme, is capable of metabolizing nicotine to cotinine in vitro (Murphy et al. 2005), but likely contributes little to nicotine's systemic levels in vivo, analogous to human CYP2A13. Instead, rat CYP2B1 and CYP2B2 are the major enzymes responsible for the conversion of nicotine to cotinine (Nakayama et al. 1993), although with less activity compared to human or mouse CYP2A. Thus, the use of CYP2B, versus a CYP2A enzyme, likely contributes to a smaller role of nicotine C-oxidation in rats compared to humans (Kyerematen et al. 1988a). There are few examples of interstrain variability in nicotine disposition kinetics or metabolism in rats. Lewis rats have been shown to have faster systemic clearance of nicotine compared to Fischer rats (Sziraki et al. 2001), and there is some evidence of different capacity for both total nicotine metabolism and C-oxidation activity between four strains, namely Sprague-Dawley, Long Evans, Fisher, and Wistar (Kyerematen et al. 1988b). No intra- or interstrain genetic polymorphisms in rat *CYP2B* have been identified. In general, there is little evidence in the literature for a genetic difference between rat strains with respect to nicotine metabolism. Because rat hepatic CYP2A enzymes do not appreciably metabolize nicotine, and because their urinary metabolite profile seems to differ from humans, this species may not be an ideal model for human nicotine metabolism.

6 Conclusions

The molecular genetics of human nicotine metabolism includes multiple polymorphic catalytic enzymes. Nicotine disposition kinetics and metabolism are highly variable between individuals and ethnic groups. Twin studies have indicated that the majority of this variance is genetic in origin, although environment influences may also contribute to the variation. Genetic studies have demonstrated a clear role of *CYP2A6* polymorphisms in both in vivo and in vitro nicotine C-oxidation. To date, this enzyme makes the most sizable contribution to the wide range of nicotine metabolism observed in humans, compared to other known influences. Still, even in persons with no detected *CYP2A6* genetic variants, there remains a substantial range of nicotine metabolic capacities. There are many molecular sources that may introduce variability to nicotine metabolism through genetics, such as the proteins implicated in CYP2A6 regulation or enzymes such as AOX1, UGTs, and FMO3.

Smoking is a complex behavior, and the environmental and genetic influences are equally complex. However, the biological rationale that links rates of nicotine metabolism and smoking is fairly simple: slower nicotine metabolism requires less frequent self-administration. The balance of evidence has become clear; the *CYP2A6* genotype is associated with levels of cigarette consumption. Several earlier studies did not confirm these findings; however, they may have been confounded by then-unknown *CYP2A6* alleles, many of which had dissimilar outcome phenotypes, or they may have simply been underpowered. As genotyping methods evolve, we expect the relationships between *CYP2A6* genotype, nicotine metabolism, and smoking behaviors to be further clarified. Today, significant advances have been made in understanding the effect of *CYP2A6* genotype on smoking behaviors, cessation success, cancer risk, and nicotine levels obtained from pharmaceutical sources.

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Sex Differences in Nicotine Action

Sakire Pogun and Gorkem Yararbas

Contents

Abstract Accumulating evidence suggests that the antecedents, consequences, and mechanisms of drug abuse and dependence are not identical in males and females and that gender may be an important variable in treatment and prevention. Although

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there has been a decline in smoking prevalence in developed countries, females are less successful in quitting. Tobacco use is accepted to be a form of addiction, which manifests sex differences. There is also evidence for sex differences in the central effects of nicotine in laboratory animals. Although social factors impact smoking substantially in humans, findings from nonhuman subjects in controlled experiments provide support that sex differences in nicotine/tobacco addiction have a biological basis. Differences in the pharmacokinetic properties of nicotine or the effect of gonadal hormones may underlie some but not all sex differences observed. Laboratory-based information is very important in developing treatment strategies. Literature findings suggest that including sex as a factor in nicotine/tobacco-related studies will improve our success rates in individually tailored smoking cessation programs.

1 Introduction

Drug abuse has been considered to be a male problem, and subsequently research on addiction has primarily been conducted on male subjects. Although studies on smoking behavior can be considered an exception in this regard, most of the experimental nicotine research on nonhuman subjects had not included sex as a factor until recently. There have been excellent reviews on sex/gender differences in observed nicotine/tobacco addiction, mainly concentrating on a specific aspect such as smoking cessation or discrimination (Perkins 1995, 1999, 2001; Gritz et al. 1996; Benowitz and Jacob 1997; Toneatto et al. 1992; Shiffman and Paton 1999; Lynch et al. 2002; Pauly 2008). The current chapter intends to review the literature findings on sex differences comprehensively, covering different aspects of nicotine/tobacco addiction in both clinical and experimental studies on human and nonhuman (rodent) subjects. Studies where sex differences have been depicted will be emphasized.

1.1 Why are the Sexes Different?

Males and females are different not only in reproductive function but also in brain structure and function, cognition, and behavior including addiction. Sex differences in brain and behavior result from complex reciprocal interactions between genes, gonadal sex, hormonal sex, effects of hormones on the brain (activational, organizational, and trophic), experience, learning, social, and other environmental influences (Pogun 2001). Human and nonhuman brains are evolved systems; therefore they are organized according to underlying evolutionary logic. Adaptive problems a species faced during evolutionary history provide insight into the functional organization of their brains. Through natural selection, the environment selects traits of individuals that enhance survival. On the other hand, sexual selection results in spreading of

traits that are narrowly restricted to the mating context, i.e., competition of one sex for the opposite sex. The ecological problems that confront males and females are largely similar, whereas the mating problems they face can be quite different. Differences in reproductive roles and division of labor result in the selection of different skills and behaviors in males and females. The human brain has elaborate programming capacity that makes some behaviors easier to learn and more rewarding than others. The brain's reward systems have substantial impact on the evolutionary success of a species and, as briefly mentioned above, evolutionary pressures on males and females are different.

There is strong evidence that male and female brains are 'programmed' differently. Sex differences in many nonreproductive behaviors such as aggression, pain and taste sensitivity, food intake and body weight regulation, the learning and retention of certain kinds of mazes, avoidance responses, taste aversion, and performance on certain schedules of reinforcement have been described in rodents for at least three decades (e.g., Beatty 1979). The role of gonadal hormones has been established in some sexually dimorphic behaviors, but not all. Modification of nonsexual behavior during the estrus cycle has also been studied extensively in rodents (e.g., Burke and Broadhurst 1966). Sexual dimorphism in the cholinergic system and the hypothalamic-pituitary-adrenal (HPA) axis has been well documented (reviewed by Rhodes and Rubin 1999). Gender differences observed in the incidence of many neuropsychiatric disorders, including addiction, provide further evidence regarding the significance of the issue and necessitate further research. In fact, in a recent article, Wetherington (2007) explains that in the earlier days, the National Institute of Drug Abuse (NIDA) study sections demanded a heavy burden of proof from drug abuse researchers who proposed to study sex differences, but with the recent awareness of the importance of sex differences in drug abuse, the burden of proof is shifting to defending why sex–gender differences should not be studied.

There are differences in vulnerability to addiction in general, and there is growing evidence that this difference is particularly pronounced in psychostimulant abuse (e.g., Brady and Randall 1999; Brecht et al. 2004). In human subjects, although the dissimilar influence of social and environmental pressures on males and females cannot be overlooked, biological factors are also evident and suggest similarities to the differences observed in animal models of addiction.

1.2 Epidemiological Findings

Tobacco use continues to be a major cause of preventable death. Although the number of smokers is gradually decreasing in developed countries, the remaining hard-core smokers are becoming harder to treat. Additionally, the decline has been less pronounced in women than in men (Fiore 1992). Another important concern for society is adolescent smokers. In the USA, in 2006, tobacco use among males above the age of 12 was higher than females (36.4 vs. 23.3%). On the other hand, the rate of

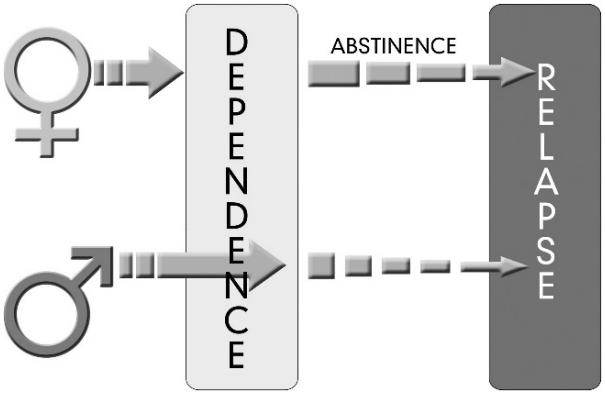

Fig. 1 Simplified diagram of sex differences in nicotine addiction. Considering both human smoking conditions and nicotine administration in animals, there are consistent sex differences, specifically during transition from use to dependence and during quit attempts, withdrawal, and relapse. Females take a shorter time to become addicted than males, make fewer attempts to abstain, and have shorter abstinent periods. Finally, females relapse more than males, indicating lower success in quitting

current cigarette smoking between ages 12–17 did not show a significant sex difference; nevertheless females had slightly higher scores than males (SAMHSA 2007).

Women have greater vulnerability for smoking-related diseases (specifically myocardial infarction and lung cancer) than men, but are less successful in quitting smoking (Harris et al. 1993; Zang and Wynder 1996; Thun et al. 2002; Henschke and Miettinen 2004; Henschke et al. 2006). Men benefit from nicotine replacement therapy more than women (reviewed by Perkins 2001). A recent meta-analysis of nicotine versus placebo patch studies has shown a significantly better response to nicotine in men than women (Perkins and Scott 2008).

Studies on rodents point to similar sex differences, suggesting the involvement of underlying sexual dimorphisms in biology. Females may take a shorter time to become dependent than males, and they make fewer quit attempts and can stay abstinent for shorter periods than males; the rate of relapse is higher in females than males (Donny et al. 2000; Perkins 2001; Pogun 2001). Figure 1 summarizes the sex differences observed in tobacco/nicotine addiction.

2 Nicotine Metabolism

There are confirmed sex differences in some biological parameters such as body weight, body fat, plasma volume, gastric emptying time, plasma protein levels, cytochrome P450 activity, drug transporter function, renal processes, and excretion activity; all these factors underlie differences in pharmacokinetics (bioavailability, distribution, metabolism, and elimination) of drugs (see chapters by Benowitz, and by Mwenifumbo and Tyndale, this volume). Nicotine is no exception in this regard.

In pioneering experimental studies, Rosecrans and Schechter (1972) showed that following nicotine injections, nicotine levels rose faster in female CD rats than males; this effect was particularly prominent in the cortex. It was concluded that female rats are more chemically and behaviorally sensitive to nicotine, relative to males (Rosecrans 1971; Rosecrans and Schechter 1972).

The metabolism and pharmacokinetics of nicotine are influenced by genetic (strain, gender) factors, which can affect the induction status of nicotine metabolizing enzymes (for a review, see Kyerematen and Vesell 1991). Sex differences have been found for the urinary nicotine metabolite profile of male and female rats (Schechter and Rosecrans 1972; Schepers et al. 1993). When rats are given a single dose of nicotine, females have lower plasma cotinine concentrations because urinary recoveries of nicotine are higher in females and the urinary output of nicotine metabolites is higher in males. These findings were interpreted to reflect a reduced rate of nicotine metabolism and a larger volume of distribution females. Overall, male rats were reported to metabolize nicotine faster than female rats after an acute intravenous (IV) injection (Kyerematen et al. 1988).

Recent studies do not comply with the earlier reports briefly summarized. Unlike the results of acute nicotine administration described above, in Sprague-Dawley rats, 24 h after termination of chronic nicotine administration (15 days, once daily, 0.6 mg kg^{-1}, s.c.), cotinine levels were not different in plasma from male and female rats, but nicotine levels were significantly lower in females than males, implying a shorter half-life of nicotine in females than in males (Koylu et al. 1997). Similar results are reported in adolescent mice. When given two-bottle free choice nicotine at different doses during the periadolescent period, female mice consumed more nicotine (mg kg^{-1}) than males but no sex difference was observed in blood cotinine levels, which correlated with nicotine dose (Klein et al. 2004b). The authors attribute the sex differences in nicotine intake, despite similar cotinine levels, to sex differences in nicotine pharmacokinetics during adolescence.

Harrod et al. (2007) administered IV nicotine to intact and gonadectomized male and female rats for 14 days, collected trunk blood 1 min after the last injection when arterial plasma nicotine levels are highest, and measured plasma nicotine levels using gas chromatography. The results indicate that female rats have significantly higher nicotine levels than males following 14 days of nicotine injections and depict an interaction between sex and gonadectomy, suggesting a role for gonadal hormones on nicotine metabolism. In other words, although intact female rats have higher plasma nicotine levels than males, gonadectomy lowers nicotine levels in females and elevates levels in males resulting in similar plasma nicotine levels. On the other hand, Donny et al. (2000) studied arterial and brain levels of nicotine following IV nicotine administration in rats that previously self-administered the drug, but did not depict significant sex differences. Route and schedule of nicotine administration and timing of sample collection may underlie the apparent differences between the studies mentioned.

Men and women regulate their smoking differently and vary in susceptibility to nicotine addiction. Earlier data from human subjects suggested that male smokers metabolize nicotine more quickly than females (Benowitz and Jacob 1984).

A similar pattern was observed among nonsmokers when they were given nicotine (Beckett et al. 1971). Although women smokers take in less nicotine per cigarette, it was proposed that since they metabolize nicotine more slowly than males, nicotine levels in the body for a given number of cigarettes per day were similar in male and female smokers (Benowitz and Jacob 1997). However, similar to the case of rodent experiments, recent reports on human subjects do not agree with these earlier findings. In a study by Zeman et al. (2002), although there were no differences between male and female smokers regarding the number of cigarettes per day or CO levels, females had lower blood nicotine levels than males.

Cytochrome p450s (mainly CYP2A6) metabolize nicotine; if CYP2A6 activity is high, nicotine is quickly metabolized and fast metabolism is usually related to high nicotine dependence (Tyndale and Sellers 2002). As reviewed by Mwenifumbo and Tyndale (this volume), CYP2A6 and CYP2B6 activities are higher in women than men. Recently, Benowitz et al. (2006) studied nicotine metabolism in twins and found that premenopausal women metabolized nicotine and its metabolite cotinine faster than men. Further analyses indicated that women needed estrogen to metabolize nicotine because metabolism in postmenopausal women was similar to men, and because women using estrogen-only oral contraceptives metabolized nicotine faster than women using progesterone-only contraceptives.

Rodent data, on the other hand, is somewhat different from observations in humans. Siu et al. (2006) studied the variation of CYP2A5, the mouse homolog of human CYP2A6, in different mouse strains that self-administer different amounts of oral nicotine. Males in the strain that were high nicotine consumers had more CYP2A5 protein and metabolized nicotine faster. However, female mice in both the high- and low-nicotine consuming strains did not show marked differences in nicotine metabolism or CYP2A4/5 protein levels. The results suggested that only among male mice, is high CYP2A5 activity correlated with faster nicotine metabolism.

In summary, although there have been contradictory reports in related literature, as well as discrepancies in data from humans and rodents, recent studies point to a higher nicotine metabolism in females than males.

3 Locomotor Activity

Studies that will be summarized in this section are on rodents.

Female rats are more active than males in tests of locomotion (Wang 1923; Beatty 1979; Burke and Broadhurst 1966; Cronan et al. 1985; Rodier 1971; van Haaren and Meyer 1991; Kanyt et al. 1999; Booze et al. 1999, Harrod et al. 2004). This sex difference is markedly reduced in gonadectomized female rats, suggesting a role for ovarian hormones (Kanyt et al. 1999; Booze et al. 1999). In a pioneering study, Rosecrans (1972) had shown that nicotine facilitated spontaneous locomotor activity in females, but had no effect in males.

The effects of nicotine on spontaneous locomotor activity in rats are complex and include both stimulant and depressant actions (Schwartz and Kellar 1985). Systemically applied nicotine increases extracellular dopamine (DA) concentrations in the nucleus accumbens (NAC), acting through the ventral tegmental area (Nisell et al. 1994). Nicotine infusions into the ventral tegmental area produce locomotor activation (Reavill and Stolerman 1990), and this effect can be weakened by lesioning the ascending mesolimbic DA pathway (Clarke et al. 1988). These observations indicate that the effect of nicotine on locomotion is through the dopaminergic system, which is substantially modulated by the hormonal environment. Estrogens and progesterone can modulate the function of DA systems in a complex manner. Estrogens can also enhance nicotine-induced DA release in striatal slices prepared from the brains of ovariectomized female, but not male, rats (Dluzen and Anderson 1997). Along the same lines, estrogens increase the binding of the nicotinic ligand [^{125}I]–bungarotoxin in the suprachiasmatic nucleus (Miller et al. 1984). Acetylcholine-evoked currents through the nicotinic ACh receptor ($\alpha4\beta2$) are inhibited by progesterone and potentiated by estradiol (Valera et al. 1992; Curtis et al. 2002).

Kanyt et al. (1999) examined the effects of sex and ovarian hormones on the effects of nicotine on locomotion in a series of experiments using male and female hooded rats. Female rats displayed higher locomotion than males. Acute nicotine reduced locomotion, and this effect was slightly larger in females than males. Significantly more reduction in spontaneous locomotor activity in female than in male rats by nicotine has been reported in other studies (Craft and Milholland 1998; Cheeta et al. 2001). On the other hand, an earlier study reports lower sensitivity of females to nicotine's motor depressant effects than males (Hatchell and Collins 1980).

Chronic nicotine administration (21 days) produced a similar, gradual increase in activity in both sexes, again with greater activity in females than males, but no interaction of nicotine effects with sex was observed (Kanyt et al. 1999). Experiments comparing ovariectomized rats and ovariectomized rats receiving hormone replacement (17-β-oestradiol and progesterone) provided clear evidence for the enhancement of the chronic locomotor-activating effect of nicotine by ovarian hormones (Kanyt et al. 1999). The most likely mechanism for this effect involves an interaction of female hormones with the mesolimbic DA system through which the locomotor activation is mediated.

A few studies have investigated the effect of nicotine on locomotor activity during the adolescent period. Elliott et al. (2004) has observed greater increases in activity in female rats than in males at lower doses of nicotine injections. On the other hand, Faraday et al. (2001) report higher activity in adolescent males than females when nicotine was administered at a single dose ($12\,\mathrm{mg\,kg^{-1}}$ per day) with mini pumps; during cessation, increased sensitivity to nicotine and thereby increased locomotion persisted in males as well. Again using osmotic mini pumps, but at a lower dose ($6\,\mathrm{mg\,kg^{-1}}$ per day), Trauth et al. (2000) report decreased locomotor activity in females compared to males.

In summary, although the results on adolescent exposure are somewhat contradictory, females are apparently more sensitive than males to the effects of nicotine on locomotor activity, and the ovarian hormones are likely to underlie this greater responsivity.

4 Reward Systems

4.1 Rodent Studies

As reviewed by Balfour (see chapter 8 in this volume), the mesolimbic DA hypothesis of psychostimulant dependence (e.g., Wise and Bozarth 1987) has been supported by many studies. Nicotine increases extracellular DA in mezolimbic (Damsma et al. 1989; Marshall et al. 1997; Nisell et al. 1996; Pontieri et al. 1996), nigrostriatal (Imperato et al. 1986; Benwell and Balfour 1997), and mezocortical (Toth et al. 1992) regions. The effect of systemic nicotine is more pronounced in the NAC than in the striatum (Di Chiara and Imperato 1988).

Sex differences exist in the regulation of central dopaminergic neurotransmission (Becker and Ramirez 1980; Becker et al. 1984; Dluzen and Ramirez 1990; Pilotte et al. 1984). Young and aged female rats have higher striatal DA and homovanillic acid (HVA) levels than males; aging reduces DA and HVA in males but not in females (Dorce and Palermo-Neto 1994). In females, the activity of the dopaminergic system varies with oscillating levels of ovarian hormones. Female rats have higher extracellular striatal DA concentration during the proestrus and estrus phases of the cycle than in diestrus or following ovariectomy (OVX). Castration of male rats does not have any effect on extracellular DA concentrations. Endogenous ovarian hormones, but not testicular hormones, modulate extracellular striatal DA concentrations in rats (Xiao and Becker 1994).

Female rats appear to be more sensitive than males to the toxic and reinforcing effects of psychostimulants that increase DA levels in the synaptic cleft; females exhibit greater behavioral responses and sensitization as well (Becker 1999; Becker et al. 2001; Morishima et al. 1993; Dalton et al. 1986; Roberts et al. 1989).

Ethanol, nicotine, caffeine, and phencyclidine stimulate both locomotor activity and dopamine turnover (Wise 1987). Sex differences in DA outflow in the NAC has been shown in response to ethanol. Female rats have a greater ethanol-induced DA outflow, as measured by microdialysis in the NAC, than males and this sexually dimorphic response is regionally specific as it is not observed in the striatum; additionally, females consume more alcohol (Blanchard et al. 1993).

As mentioned in the previous section, we have observed greater impact of nicotine on locomotor activity in female than in male rats (Kanyt et al. 1999). The psychostimulant theory of addiction also proposes that the locomotor stimulant and rewarding effects of addictive drugs have a common neuronal substrate, although there are some studies that do not support this hypothesis (Carr et al. 1988; Villegier et al. 2006). Species differences or methodological differences in the studies may underlie the discrepancies.

There are sex differences in DA receptor regulation and density through development. Male rats have higher densities of D1 receptors in the NAC during development than females and this difference is maintained in adulthood (Andersen et al. 1997). Male rats also show a greater increase in striatal D2 receptor density at the onset of puberty, but these receptors are substantially pruned by adulthood (Andersen and Teicher 2000). The sex differences in DA receptor densities and regulation during development may underlie sex differences in vulnerability to drug abuse.

Harrod et al. (2004) studied DA transporters and D1, D2, and D3 receptors following repeated intravenous nicotine administration in male and female rats. No sex differences were depicted in the number of D1 or D2 receptors in either the striatum or the NAC, but female rats had increased number of DA transporters in both the core and shell of the NAC and decreased density of D3 receptors in the shell of NAC, compared to males. Nicotine-induced changes of DA transporters and D3 receptors are reported to be partly responsible for increased behavioral sensitization measured by locomotor activity in female rats.

4.1.1 Effect of Gonadal Hormones

Ovarian hormones modulate the function of DA systems in a complex manner (Saigusa et al. 1997; van Hartesveldt and Joyce 1986) and subsequently modify central nicotinic cholinergic transmission systems and the behavioral effects of nicotine. Hormonal effects on DA release, which have been observed in the absence of drug perturbations, may also influence the effects of nicotine. In female rats, estrogen increases DA release in the striatum and NAC through a G-protein-coupled external membrane receptor and enhances DA-mediated behaviors. In male rats, neither estrogen nor testicular hormones have an effect on mesolimbic DA systems. The greater sensitization of female rats to hormonal effects on the mesolimbic DAergic system may underlie the sex differences in susceptibility to addiction to the psychomotor stimulants (Becker 1999) as well as nicotine.

Booze et al. (1999) have shown that while repeated intravenous nicotine administration induced behavioral sensitization in male and female rats, female rats exhibited increased sensitivity to repeated nicotine, relative to males. Furthermore, repeated nicotine administration did not interfere with intact female vaginal cytology, or produce persistent vaginal estrus, estrus acyclicity, or changes in body weight. The peak arterial nicotine concentrations were similar in male and female rats.

4.2 Human Studies

In human subjects, Laakso et al. (2002) reported higher DA synthesis capacity in women than men on the basis of higher [^{18}F] fluorodopa uptake in the striatum. Staley et al. (2001), using positron emission tomography (PET) imaging, found

higher DA and serotonin transporter availability in females than males. On the other hand, a recent PET study on healthy human subjects using [^{11}C] raclopride to image DA D2 and D3 receptors reports greater DA release in the ventral striatum, anterior putamen, and anterior and posterior caudate nuclei in men than women in response to an amphetamine challenge, whereas no sex differences in DA receptor binding were depicted at baseline levels. This response was accompanied by a greater positive effect of amphetamine in men although no sex differences were observed in the levels of cortisol, estradiol, progesterone, total testosterone, and free testosterone (Munro et al. 2006). Whether this response to amphetamine can be generalized to psychostimulants, including nicotine, warrants further investigation. Furthermore, the sex differences reported by Munro et al. (2006) in human subjects were observed in the associative regions of the striatum, whereas the preclinical studies on addiction involve the sensorimotor areas (Martinez et al. 2005)

Overall, the central dopaminergic systems mediating reward appear to be more active in females than males, notwithstanding the fact that there may be exceptions under some conditions.

5 Genetics

Strain differences in responses to nicotine are well documented in rodents, albeit these studies have concentrated on species differences using male animals (e.g., Collins et al. 1988). If there are strong strain differences in response to nicotine suggesting genetic vulnerability to or protection from nicotine addiction, sex is most likely another factor that could impact responses to nicotine as well.

Twin and adoption studies indicate that genetic factors account for 55% of smoking initiation and 61% of persistence. The heritability of initiation of smoking is reported to vary by gender, with women showing greater vulnerability for heritability regarding initiation of smoking (66% in women vs. 49% in men); on the other hand, the heritability of persistence or maintenance of smoking does not show sex differences and is 61% in both sexes (Hamdani et al. 2006).

5.1 Candidate Genes

Research on candidate genes for smoking or tobacco use has focused on dopaminergic neurotransmission, mainly because of the well-established effect of nicotine on the mesolimbic reward system. Nicotine metabolism is another aspect that is being studied extensively (see chapter 9 by Mwenifumbo and Tyndale, this volume). The serotonergic system is also receiving attention, specifically because of the interaction between nicotine use and anxiety and depression. The results of these studies point to sex differences. As reviewed by Mwenifumbo and Tyndale, the activity of the cytochromes P450 (CYP2A6 and CYP2B6) is significantly higher in women than men and higher metabolism is related to nicotine dependence.

Haplotypes are a group of closely linked alleles (genes or DNA polymorphisms) that are inherited together on a single chromosome; this information is very valuable for investigating the genetics behind common diseases. The catechol-*O*-methyltransferase (COMT) gene has impact on central dopaminergic neurtotransmission and thereby reward. Allelic variants within the COMT gene point to individual differences, including sex, in vulnerability to nicotine dependence. For example, the A–G–T haplotype is protective only in African American females; the T–G–T haplotype is protective only in European American males; and the T–A–T haplotype is associated with high risk in European American males. The two protective haplotypes are associated with low COMT enzyme activity (Beuten et al. 2006).

Brain-derived neurotrophic factor (BDNF) interacts with both dopaminergic and serotonergic neurotransmission and subsequently also has impact on the reward systems. A significant linkage to nicotine dependence has been reported in a genomic region on chromosome 11 where the BDNF gene is located. However, in further single nucleotide polymorphism (SNP) analyses, significant associations with nicotine dependence were observed only in males with European American ancestry, but not in females, suggesting a sexually dimorphic association between BDNF and nicotine dependence (Beuten et al. 2005).

Li et al. (2005) studied the association of allelic variants of the nAChR a4 subunit gene (CHRNA4) with nicotine dependence in African and European Americans. A common haplotype (C–G–G; 53.4%) was identified in African American females, which protected against nicotine dependence, while in the same group, another haplotype (C–A–A; 14.2%) formed by the same SNPs showed a positive association with nicotine dependence. These findings suggest the existence of ethnic and gender specificity in the association of CHRNA4 with nicotine dependence. The same study did not detect a significant involvement for nAChR b2 subunit gene (CHRNB2) (Li et al. 2005).

Overall, genetic vulnerability to or protection from nicotine/tobacco addiction shows significant sex differences, which may underlie the differences observed in addiction patterns between males and females, ranging from initiation of smoking to success in quitting.

6 Development: Prenatal and Adolescent Exposure

The effects of nicotine through different stages of development are not the same. Prenatal exposure to nicotine interferes with neural development and synaptic function and increases the probability of cognitive deficits in the offspring. Moreover, prenatal exposure impacts the effects of nicotine (rodents) or tobacco smoking (humans) during adolescence. Adolescence is a time that deserves special emphasis in addiction research because there is a surge of sex hormones that may have organizational effects on brain structure and function, and also because during this period adolescents are more vulnerable to social and environmental pressures, specifically of peers. The effects of nicotine on the adolescent brain are different from its effects

on the adult brains and are sexually dimorphic. Not only prenatal or adolescent but also adult exposure to nicotine produces persistent changes in the central nervous system (CNS), which render the subject vulnerable to nicotine addiction and relapse after quitting.

6.1 Rodent Studies

Results obtained from rodents suggest that whereas males are more sensitive to developmental changes, females are not totally spared regarding deficits in the cholinergic system; nicotine exposure during any developmental period produces persistent functional changes in specific brain regions at later stages. However, effects at earlier stages are more severe (Slotkin et al. 2007; Abreu-Villaca et al. 2004). When mothers were given oral nicotine during pregnancy and offspring were tested for nicotine preference during the periadolescent period, male offspring of mothers exposed to nicotine showed increased preference, while maternal nicotine exposure did not alter nicotine preference in female mice (Klein et al. 2003).

Prenatal nicotine impairs cholinergic and serotonergic systems in rat brain in a regionally specific manner and the effects are more pronounced in the male than the female brain (Slotkin et al. 2004, 2007; Slotkin and Seidler 2007).

Adolescent nicotine exposure results in pronounced and persistent nicotinic cholinergic receptor upregulation in male rats and hippocampal cell damage in females (Trauth et al. 1999). During and following nicotine treatment as adolescents, female rats show impaired rearing and locomotor activity, whereas males are unaffected. On the other hand, improved performance was observed in passive avoidance (Trauth et al. 2000).

Serotonergic systems are also affected by adolescent treatment in a sexually dimorphic manner. During adolescence, at doses that produce plasma nicotine doses similar to levels found in smokers, nicotine decreases serotonin receptors specifically in the cortex, in female rats. However, in the midbrain, there was an increase in males (Xu et al. 2002).

6.1.1 Effect of Gonadal Hormones

There may be different factors underlying the differential vulnerability of male and female rodents to prenatal nicotine exposure. Prenatal nicotine treatment affects fetal plasma testosterone and perinatal sexual brain differentiation in the rat. Aromatase converts androgens to estrogens and is important in sexual differentiation. The male offspring of rats that received nicotine during pregnancy showed decreased aromatase activity compared to female offspring, whereas no drug effect was seen in female fetuses and offspring. Sex differences in the developmental effect of nicotine may thus involve brain aromatase (von Ziegler et al. 1991).

Estrogen receptors, which underlie hippocampal neurogenesis and synaptic plasticity, may be protective through providing greater adaptive capacity in females (McEwen 2002; Tanapat et al. 1999). However, when rats prenatally exposed to nicotine are treated with nicotine as adolescents, the impairment extends to females as well (Slotkin et al. 2007). Furthermore, nicotine treatment during adolescence followed by withdrawal is reported to cause hippocampal cell damage resulting in behavioral deficits, mainly in female rats (Xu et al. 2003).

6.2 Human Studies

Pregnancy is a very special condition that deserves attention with regard to female smokers. A recently published 30-year progressive study reported that, in addition to the immediate health risks that are related to smoking during pregnancy (e.g., higher rates of abortion, premature placental abruption, low birth weight, malformations, and sudden infant death syndrome), when offspring try cigarettes later on in life, the odds of progressing to nicotine dependence was almost twice as much compared to offspring from nonsmoker women. Interestingly, the elevated risk of developing nicotine dependence seen in the offspring of mothers who smoked more than one pack of cigarettes was not observed in marijuana dependence, despite its similar route of administration (inhalation) and the reported association between cigarette smoking and marijuana (Buka et al. 2003).

Prenatal exposure to nicotine impairs auditory and visual attention, and this impairment is gender specific. When smoker or nonsmoker adolescents with or without prenatal exposure to maternal smoking were tested for auditory and visual-selective and divided attention, females exposed to tobacco smoke during adolescence or prenatal development showed reduced performance accuracy. Among males, marked deficits were observed in auditory attention (Jacobsen et al. 2007).

Findings from both rodent and human experiments infer sex- and region-specific biobehavioral effects of nicotine through development and suggest a role for smoking in the higher incidence of depression, especially among adolescents and females. In fact, clinical studies with human subjects note that adolescents who were exposed to nicotine in utero are most vulnerable to nicotine addiction (Cornelius et al. 2000; Kandel et al. 1994; Niaura et al. 2001). Future studies on the epigenetic effects of nicotine will enhance our understanding of prenatal or adolescent nicotine exposure.

7 Nicotinic Receptors

Nicotine is unique in causing upregulation of nAChRs following chronic treatment in male rats. In rodents, chronic nicotine administration increases nicotinic receptor sites in several brain regions (Marks et al. 1985; Schwartz and Kellar 1983; Ksir et al. 1987) and this upregulation corresponds to the sensitization

of locomotor stimulant action of acute nicotine doses after chronic administration (Ksir et al. 1987). The time course of receptor upregulation and the persisting cognition-enhancing effects of nicotine are similar (Levin et al. 1992). This upregulation shows sexual dimorphism: chronic nicotine treatment causes nicotinic receptor upregulation in the brains of male but not female rats. Female rats have higher densities of nicotinic acetylcholine (nACh) receptors, and nicotine treatment ($0.6\,mg\,kg^{-1}$, s.c.) for 15 days causes an upregulation of ^{3}H-cytisine binding in the brains of male rats but not in females. The observed upregulation in male rats does not persist after a withdrawal period of 20 days. (Koylu et al. 1997). Sex differences in upregulation of brain nicotinic receptors (nAChRs) by chronic nicotine treatment have also been demonstrated in vivo in mice, using a specific radioligand for nAChRs, [^{125}I] IPH. Although binding was increased in all brain regions studied, a significant sex difference was depicted with male animals showing a more pronounced increase than females (Mochizuki et al. 1998). On the other hand, when rats self-administer nicotine, no sex differences in nicotinic receptor upregulation is observed (Donny et al. 2000). In view of these studies, the route of nicotine administration may be an important factor in inducing nicotinic receptor regulation in rodents.

In human smokers, smoking (nicotine and other constituents of tobacco) increases dopamine (DA) and serotonin (5-HT) levels in brain and may alter DA and 5-HT transporter expression and function. DA and 5-HT transporters are modulated by sex steroids, suggesting sex differences in transporter function in smokers. Using single photon emission computed tomography (SPECT) and [^{123}I] β-CIT to label DA and 5-HT transporters in brain, brainstem [^{123}I] β-CIT uptake was found to be modestly higher (10%) in smokers than nonsmokers. Analyzing data regardless of smoking status revealed higher uptake in the striatum (10%), diencephalon (15%), and brainstem (15%) in females than in males. Although brainstem uptake was 20% higher in male smokers and only 5% in female smokers compared to nonsmokers, sex x smoking interaction was not significant. The results demonstrate higher DA and 5-HT transporter availability in females than males and no overall effect of smoking with the exception of a modest elevation in brainstem 5-HT transporters in male smokers. Brainstem 5-HT transporters may be regulated by smoking in a sex-specific manner (Staley et al. 2001).

Overall, a limited number of experiments on rodents suggest that the upregulation of nicotinic receptors by nicotine treatment is observed in males but not females. On the other hand, nicotine self-administration has the same upregulation effect on both male and female rats. In human smokers, DA and 5-HT transporter availability appears to be higher in females than males although the difference is not substantial.

8 Consummatory Behavior and Body Weight

The effect of smoking on body weight has been one of the major obstacles in smoking cessation programs. Although the concern of women about weight gain is greater than men, and women often report that they smoke cigarettes to avoid weight gains

and that they relapse after abstaining from tobacco to prevent weight gains, empirical evidence does not totally support this impression. The greater impact of social and cultural pressures about physical appearance on women is likely.

The thermogenic effect of nicotine is more prominent in males, and physical activity level enhances the acute metabolic effect of nicotine (Perkins et al. 1994b, 1991). Nicotine does not reduce hunger and eating acutely and does not have an anorectic action; in this regard, no difference between males and females is reported (Perkins et al. 1994a, 1992a). Klein et al. (2004a) studied the leptin responses in smoker and nonsmoker males and females; although leptin and reported hunger levels were different between the groups, smoking status was not related to leptin levels in either sex.

Animal and human studies have demonstrated that the self-administration of palatable foods, especially sweets, increases after nicotine deprivation (Hughes et al. 1991; Ogden and Fox 1994; Spring et al. 2003). In abstinent human smokers, the reward value of carbohydrate-rich snack foods were higher in females than males, suggesting that food and nicotine may be substitutable rewards, especially for females (Spring et al. 2003).

Nicotine reduces food consumption and increases metabolic rate. Quitting smoking results in weight gain in male and female smokers (Hill et al. 2000; Pomerleau et al. 2000) and there is an inverse relationship between nicotine and body weight (Klesges et al. 1991). However, the persistence of weight gain (at 10 weeks) is observed only in women and is attenuated by nicotine replacement (Hill et al. 2000). The impact of physical activity on the acute thermogenic effect of nicotine is more pronounced in male smokers than females, possibly resulting in differences in body weight changes during tobacco smoking and cessation (Perkins et al. 1991). The effect of smoking on body weight has apparently been perceived as a significant factor in the decision to continue smoking in women, and tobacco companies have used it in their advertisements and the design of their cigarettes targeting women, extensively. Seventy-five percent of women and 35% of men cannot tolerate more than 2–3 kg of weight gain after quitting (Pomerleau and Kurth 1996). Cognitive behavioral therapy to reduce weight concerns improve smoking cessation outcome in weight-concerned women (Perkins et al. 2001b).

To exclude the effect of cultural factors and cognitive concerns about body weight, Grunberg et al. (1986) studied the effect of nicotine administration on weight gain in male and female Sprague-Dawley rats. Greater effects of nicotine administration and cessation on body weight and eating behavior was observed in female than in male rats, suggesting either differences in sensitivity to nicotine or differences in the time course of nicotine's effects. In a follow-up study, long-term effects of nicotine cessation on body weight were evaluated in female and male Sprague-Dawley rats with nicotine or saline treatment for 16 days. Body weight, food consumption, and water consumption were measured before, during, and after nicotine administration and additionally for 4 months after cessation of nicotine. An inverse relationship between nicotine and body weight was observed as well as an inverse relationship between nicotine and general consummatory behavior for females but not for males. The body weight of females that had received nicotine

were indistinguishable from controls up to 4 months after cessation of nicotine, while the body weight of males that had received nicotine remained lower than controls (Grunberg et al. 1987). This study has demonstrated a long-term effect of nicotine treatment on body weight in males but not in females. In another study, similarly, nicotine withdrawal produced significant increase in food consumption and weight gains, while nicotine administration decreased food consumption and inhibited weight gain in rats. Larger effects were obtained for males than for females (McNair and Bryson 1983). Levin et al. (1987) examined the effects of chronic nicotine and withdrawal on food and water consumption and body weight in female rats and found that changes in weight gain were accompanied by changes in food consumption; furthermore, nicotine withdrawal caused hyperphagia and hyperdipsia.

Overall, although studies with rodents suggest an inverse relationship between nicotine and body weight in both sexes, this effect is more pronounced in female rats than males. With some exceptions, studies suggest that females are more vulnerable to weight gain after quitting nicotine. Additionally, while systemic nicotine decreases food intake in both sexes, this effect (reduced meal sizes) is not related to sex hormones in rodents (Blaha et al. 1998).

Both human and rodent studies suggest that the persistent effect of nicotine on body weight is slightly higher in females than males. In humans, controlling weight by smoking, concern about weight gain after quitting, and thereby continuing to smoke are major problems among women smokers.

9 Self-Administration

Male and female rats self-administer nicotine, but female rats are reported to acquire self-administration at lower doses and faster than males, suggesting that motivation to obtain nicotine is higher in females than males. Females also reach higher break points on a progressive ratio, indicating that they are willing to pay more to obtain nicotine. Females have shorter latencies to earn their first infusion and demonstrate higher rates of both inactive and timeout responding. Estrous cycle does not have an effect on self-administration (Donny et al. 2000). A similar pattern is observed in adolescent mice when nicotine is presented orally and the animals have 24 h access to both saccharin-only and nicotine-containing solutions: females consume more nicotine than males (Klein et al. 2004b). The faster nicotine metabolism in females than males may partially underlie the observed effects.

The reinforcing effect of nonpharmacological stimuli on operant responding for nicotine (Caggiula et al. 2001) suggests that environmental cues may play an important role in nicotine addiction (Caggiula et al. 2002). Male and female rats self-administer nicotine in the absence or presence of nonpharmacological stimuli; for example they acquire lever pressing in the absence of visual stimuli. However, when the active lever is combined with a visual cue, female rats respond more than males, specifically at higher nicotine doses. On the other hand, female rats also respond

more than males on the nonreinforced lever. Considering that the lever is also a cue (like the visual stimulus) for the animal, greater responding suggests that responding for nicotine per se may be smaller in female than in male rats (Chaudhri et al. 2005).

9.1 Non-nicotine Stimuli Associated with Nicotine or Smoking

Studies from A. Caggiula's laboratory on nicotine self-administration in rodents (Caggiula et al. 2002; Donny et al. 2003; Chaudhri et al. 2005, 2006a, b, 2007; Palmatier et al. 2006) or cigarette smoking behavior in humans (Rose and Levin 1991; Rose et al. 1993; Shahan et al. 1999) suggest that nicotine consumption is reinforced by associated non-nicotine, presumably conditioned stimuli. Nicotine replacement is perceived to be less pleasurable than smoking even when the nicotine doses are equal (Perkins et al. 1994c; Westman et al. 1996). In fact, Balfour et al. (2000) suggest that conditioned reinforcement may be a stronger influence on cigarette smoking than on other drug dependencies.

Perkins et al. (2001a) studied the influence of visual and olfactory/taste stimuli on the reinforcing effects of smoking and consummatory behavior in men and women. Women were more sensitive than men to blockade of olfactory/taste stimuli with regard to puff self-administration and hedonic ratings, suggesting that conditioned reinforcement of smoking behavior is more pronounced in women than in men. On the other hand, no sex differences were observed in other consummatory behaviors (of food or alcohol) under similar conditions. As the authors suggest, if smoking cessation programs focus on extinguishing conditioned reinforcing effects of non-nicotine smoke stimuli, better results may be obtained in women smokers, who are generally less successful than men in quitting smoking.

Non-nicotine stimuli associated with nicotine or smoking play an important role in craving as well. Cue reactivity can be measured using different approaches such as self-reports, physiological reactions, or by imaging brain activity. Niaura et al. (1998) presented different types of cues to exsmokers and obtained the greatest physiological responses with 'in vivo exposure' in both sexes. Sex differences emerged when 'affectively valenced standardized scripts depicting situations generally associated with relapse' were presented: women had greater increases in arterial blood pressure than men. Field and Duka (2004) exposed smokers to 'smoking paraphernalia' and also manipulated perceived cigarette availability before cue exposure. Smoking cues increased craving and skin conductance levels, a physiological measure of arousal, in both sexes. However, another physiological measure, salivation, depicted significant sex differences. While men responded with decreased salivation to smoking cues, women responded with increased salivation, but only when cigarettes were perceived as unavailable. In a recent functional magnetic resonance imaging (fMRI) study, women had larger cue reactivity in right putamen, bilateral cuneus, and left middle temporal gyrus, while men had greater responses in left hippocampus and left orbitofrontal cortex. Further analyses indicated that cue reactivity was correlated with negative effect and was notably observed in men (McClernon

et al. 2007). These studies suggest that although the subjective reports of craving in men and women are apparently similar, according to the study design (cues presented and responses measured), sex differences are depicted in physiological responses and the patterns of brain activity.

Smoking behavior has a strong conditioning component, and therefore smoking-related cues in human smokers and nicotine-related cues in animal self-administration studies have significant impact on the interpretation of the observed findings. Although females appear to be more sensitive to non-nicotine cues than males in both human and rodent studies, sex differences in the organization of related brain circuitry should not be overlooked.

9.2 Aversion

Nicotine is an addictive substance with rewarding and reinforcing properties. On the other hand, the autonomic responses following an acute nicotine treatment and the bitter taste of nicotine may cause aversion. This aversion may impact conditioned effects to nicotine. Rinker et al. (2008) studied possible sex differences in taste aversion induced by nicotine in rats; systemic nicotine or saline injections were paired with oral saccharine. Although nicotine did produce a weak taste aversion, no sex differences were observed, excluding the possible contribution of the aversive properties of nicotine on sexually dimorphic responses to nicotine. The authors conclude that sex differences may arise from differences in the rewarding properties of the drug.

10 Cognitive Effects

Nicotinic receptors are present in brain regions critical for cognitive function and addiction: cortex, striatum, and ventral tegmental area. Although the validity of some earlier studies on the cognition-enhancing effects of nicotine or smoking can be criticized, the cognitive effects of nicotine may be of importance in the decision whether to continue smoking (e.g., Colrain et al. 1992; Levin et al. 1992; Warburton et al. 1992; Mangan and Golding 1983; Peeke and Peeke 1984).

On average, human males and females are similar in cognitive abilities, and task-dependent sex differences are noticeable at the extremes (Kimura 1999). Performance on cognitive tasks is affected not only by differences in cognitive ability but also by cognitive style or behavioral strategy. Furthermore, cognitive strategy can be influenced by various factors including pharmacological manipulations, age, hormones, or sex.

Basal brain activity and regional brain activation levels are not the same in males and females. Fallon et al. (2005) studied brain metabolism with 2-[^{18}F] fluoro-2-deoxy-D-glucose and positron emission tomography (FDG-PET) in male and female

subjects during cognitive tasks. Under basal and placebo conditions, the activity was higher in females, especially in the forebrain regions, but nicotine had a sexually dimorphic effect and eliminated the sex differences observed.

There are several studies pointing to sex differences in problem-solving strategies. While males use an impulsive-global strategy, females prefer a reflective-sequential task-solving strategy, and therefore are slower but more accurate (Klinteberg et al. 1987). Women generally want to have more/additional information in decision making (Pratt et al. 1988). Algan et al. (1997), studied the effects of sex and nicotine (smokers vs. nonsmokers) in verbal and spatial cognitive tests. The results of the study imply that smoking has a gender-specific effect on cognitive function: it improves the performance of males in a verbal task and increases the subjective confidence of females, thereby affecting the preferred cognitive strategies for problem solving. Independent of the task type, smoking altered the 'no-response' rate (i.e., the subject cannot decide on the correct response and does not respond) uniquely in female smokers. While female nonsmokers had a higher no-response rate than males, female smokers were responding to almost all the stimuli presented in both verbal and spatial cognitive tasks. In males, no effect of smoking was observed on the no-response rate. In other words, smoking did not affect the strategy used by males in problem solving while it significantly modified it in females, shifting the female style towards the male one. This sort of modification of sex-related strategies of cognitive styles for problem solving suggests that smoking has an action on females, such that their approach to the solution of a problem is modified.

For small animals like rats, the water maze (WM) offers the possibility of making the distinction between cognitive ability and style. In a modified version of the WM place learning task, following acquisition, Kanit et al. (1998) offered the rats a choice of finding the platform by using visual or navigational cues. Rats displayed a very significant sex difference in trying to escape, and adult female rats went to the visible platform using visual cues while males searched for the hidden platform using navigational cues. However, when the animals were treated with nicotine, the cognitive strategy was totally changed for female rats only, and nicotine-treated females behaved like males, preferring the navigational strategy.

In addition to modifying cognitive strategy, nicotine can enhance performance in several tests of cognition in animals as well. Yilmaz et al. (1997) tested the effects of nicotine on active avoidance learning in male and female Sprague Dawley rats. The results provide evidence that nicotine improves cognitive function in rats during the acquisition phase of active avoidance learning trials in a dose-dependent manner. Male rats benefit from nicotine at all doses tested, whereas in females learning performance deteriorates at the higher dose of $0.6\,\mathrm{mg\,kg^{-1}}$, suggesting that nicotine pretreatment affects active avoidance in a sexually dimorphic and dose-dependent pattern. The observed effect of nicotine during the acquisition phase does not totally persist after the termination of nicotine administration: the performance of nicotine-treated male rats falls significantly below the levels of saline-treated animals during the second and the third weeks after nicotine injections were discontinued. Although ceiling effects cannot be totally precluded, when the performance of saline-treated

rats are considered, decreased performance of nicotine-treated male rats is more likely to reflect the lack of persistent drug effects. While young knockout mice lacking the β2 subunit of the nicotinic receptor do not show any difference in fear-conditioning and latent-inhibition tasks compared to wild-type, the male, aged, β2 knock-out mice, but not females, were impaired in fear conditioning (Caldarone et al. 2000).

On the other hand, cigarette smoking during pregnancy is reported to be related to cognitive deficits in the children; similar results have been obtained in rats with nicotine administration during gestation, and in a sexually dimorphic pattern. Nicotine exposure during fetal life (1–20 days of gestation) improved learning (active avoidance) in female rats but reduced it in males when tested at 60 days of age (Genedani et al. 1983). Levin et al. (1996) demonstrated that cognitive deficits induced by nicotine exposure during puberty have subtle but persistant effects, which can be augmented with noradrenergic challenge. In male rats prenatally exposed to nicotine, β-NE agonist challenge facilitated choice accuracy compared to control rats; however, in females, α-NE agonist challenge caused a significant deficit in control females but not in the females prenatally exposed to nicotine. There are substantial methodological differences between the two studies mentioned.

The studies summarized above indicate that while nicotine has sexually dimorphic effects on cognitive processes, no generalizations can be made. The effects depend on the type of test and performance measures as well as on the route and regimen of nicotine administration. Furthermore, the species, strain and age of animals, motivation, and mood would have substantial impact on the cognitive effects of nicotine.

11 HPA Axis, Anxiety, and Stress

It is established that nicotine is a sympathomimetic agent and is expected to enhance physiologic and biochemical stress responses; however, smokers' reports do not comply with this explanation. This phenomenon is referred to as Nesbitt's paradox (Parrott 1998). Does nicotine normalize behavior of subjects under stress (Acri 1994)?

11.1 Subjective Reports Versus Physiological Measures in Human Subjects

Evaluation of data on subjective reports on reasons for smoking indicates that women smoke for stress reduction and men for stimulation (Best and Hakstian 1978; Frith 1971; Spielberger 1986; Ikard and Tomkins 1973), implying that there may be gender differences regarding the motivation for smoking. However, the validity of self-reports is questionable and empirical evidence based on physiological measures does not support these observations.

In human nonsmoker subjects, an acute nicotine manipulation blocked ratings of anxiety, discontent, and aggression induced by stress exposure in females, but enhanced the same ratings in males, suggesting that smoking may be perceived as a form of stress self-medication for females (File et al. 2001). However, when the same group studied and compared smokers and nonsmokers under similar conditions, the same pattern of results was not observed (File et al. 2002). Perkins et al. (1992b) studied the 'nicotine paradox' using computer tasks in male and female nonsmokers and smokers following smoking or sham smoking (unlit cigarette). In smokers who smoked during the test, subjective stress was reduced immediately after smoking in males; stress reduction was partial in females. Cardiovascular responses were not directly related to subjective changes. These findings suggest that the stress-reducing effects of smoking may be partly gender-dependent, and dissociated from the effects of smoking on cardiovascular arousal. Furedy et al. (1999) recorded cardiovascular and electrodermal activity parameters during two-session cognitive tests in smoker and nonsmoker male and female subjects. The cardiovascular effects did not show any sex differences (i.e., heart rate was increased in all smokers after smoking). During the verbal task, smoking a cigarette increased skin resistance level in males, but decreased it in females, suggesting that the acute smoking manipulation produced relaxation in the males and arousal in the females. Along the same lines, but in a different context, another study depicted higher salivary cortisol levels in women than men and accompanying negative affect during acute nicotine withdrawal (Hogle and Curtin 2006).

These findings reflect autonomically controlled measures that are unavailable to consciousness and appear to be the reverse of several subjective reports. In contrast to some, but not all, subjective reports, nicotine appears to produce arousal in women and relaxation in men.

11.2 Rodent Experiments

Nicotinic receptors influence the HPA axis differentially in male and female rats: male rats have a greater arginine vasopressin (AVP) response, while female rats show greater adrenocorticotropic hormone (ACTH) and corticosterone (CORT) responses to nicotine (Rhodes et al. 2001a, b). Faraday et al. (2005) studied the effect of sex and genotype (Sprague-Dawley and Long-Evans) on stress responsivity (CORT and ACTH levels) and nicotine in rats. Immobilization stress increased CORT and ACTH levels in all groups except Long-Evans females, and chronic nicotine increased CORT and ACTH levels of Sprague-Dawley females only. Feeding and body weight were decreased by both nicotine and stress, but the effects were most pronounced in Long-Evans females. Muscarinic and cholinergic influences on the HPA axis were also sexually dimorphic (Rhodes et al. 2001a, b).

The effect of nicotine on anxiety is sexually dimorphic, and specifically in adolescence. Nicotine increased social interaction following social isolation in both male and female rats, indicating an anxiolytic effect. This effect was more

pronounced in females and was obtained at lower doses (fivefold) than males. The sensitivity to the anxiolytic effects of nicotine during adolescence may have impact on initiating and maintaining smoking (Cheeta et al. 2001). On the other hand, nicotine induces anxiety-like behaviors, which are augmented by ethanol-withdrawal male, but not female, rats. Ovariectomized rats behave like males, suggesting the influence of ovarian hormones (Jung et al. 2000).

A review of the literature on the effects of nicotine on stress again draws our attention to the fact that generalizations cannot be made. However, in rodents, the anxiolytic effect of nicotine appears to be more pronounced in females than males, suggesting an interaction with ovarian hormones.

12 Neuropsychiatric Disorders

There are gender differences in the prevalence and clinical presentation of many neuropsychiatric disorders. Since most neuropsychiatric disorders involve disturbances in emotional regulation and reward, Adinoff et al. (2003) have studied the responses of the limbic system to a pharmacological stimulus with SPECT. Procaine was administered to activate limbic structures, and regional cerebral blood flow was measured. In general, despite similar subjective responses following procaine injection, activation patterns in the limbic regions were sexually dimorphic in healthy men and women. In women, bilateral medial and anterior temporal regions were activated, while bilateral insular regions were activated more in men. Activation of the anterior cingulate cortex was similar in men and women.

Among the well-documented effects of nicotine on many neurotransmitter systems (reviewed by Mansvelder and McGehee 2002), modulation of the γ-aminobutyric acid (GABA) neurons to induce GABA release (Fuxe et al. 1989) deserves special attention, since GABAergic dysfunction underlies several affective disorders. Nicotine's effects on mood may be through GABAergic neurons. Epperson et al. (2005) showed that female smokers had reduced cortical GABA levels during the follicular phase of the menstrual cycle, whereas cortical GABA levels were similar in smoking and nonsmoking men. Nicotine modulation of GABA levels in women may underlie the depressive symptoms women experience during smoking cessation.

13 Smoking Cessation

There are extensive reviews focused on the gender differences in smoking cessation. Therefore the topic will be only briefly highlighted.

Although the prevalence of tobacco smoking has declined, the decline has been less pronounced in women than in men (Fiore 1992). There are gender differences in quit rates: women typically are less successful and remain abstinent for shorter

periods than men. The difference becomes even more pronounced with nicotine-replacement therapy, suggesting that nicotine can be less reinforcing compared to nonpharmacological aspects of smoking (Perkins et al. 1999; Perkins 2001; Wetter et al. 1999). Poorer outcome with pharmacotherapy, not only directed at nicotine addiction but also depression, has been reported in women than in men (Frank et al. 1988).

Overall, nicotine is often less reinforcing in women than men. Perkins (1999, 2001) and Perkins and Scott (2008). TNR attribute this sex difference to differences in nicotine's discriminative stimulus effects. In fact, the tobacco industry has been using data from their internal research on gender differences to design products specifically targeting women (see chapter 16 by Ferris Wayne, this volume). Treatment strategies that consider the sex of the subject would apparently lead to higher success rates in smoking cessation programs.

14 Concluding Remarks

There are confirmed sex differences in brain and behavior, and therefore the central effects of nicotine and in nicotine/tobacco addiction would also be expected to vary between males and females. During the past four decades, there have been substantial preclinical and clinical data pointing to sex differences in nicotine/tobacco addiction. However, there are still some discrepancies that need to be elucidated by future research. Differences in the design of the experiments, nicotine doses, routes of delivery, and controlling for the effects of hormones are some of the factors that underlie the apparent controversies. While some studies are descriptive and define observed sex differences, others have taken a more systematic approach and attempted to explain the underlying mechanisms. Sexually dimorphic pharmacokinetics that causes variance in blood/brain levels of nicotine or the effects of gonadal hormones may underlie some of the sex differences observed in nicotine/tobacco addiction. In this review, the focus has been on studies where sex differences were depicted; therefore studies where no sex differences were reported were not discussed. Biobehavioral studies including sex as a factor will help us understand nicotine/tobacco addiction better and develop more efficient and individual-based therapeutic strategies for smoking cessation.

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Part III
Nicotine Psychopharmacology

Recognising Nicotine: The Neurobiological Basis of Nicotine Discrimination

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Contents

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Abstract Drug discrimination methodology makes possible the objective, quantitative study of the perception of psychoactive drug effects in either human or animal subjects. Investigations of the nicotine discriminative stimulus complex have contributed to our present understanding of nicotine psychopharmacology by defining the origin of its effects at specific subtypes of nicotinic receptor and the role of diverse neurotransmitter systems as mediating and modulating mechanisms. The evidence strongly supports central sites as the origins of the nicotine stimulus, and these are likely to be located in the mesocorticolimbic dopaminergic neurons; the medial prefrontal cortex is primarily involved, with the Nucleus accumbens and ventral tegmental area of secondary importance, while another element of the complex stimulus may arise in the dorsal hippocampus. Additionally, it appears that interactions of nicotine with the dopamine, serotonin, cannabinoid and probably glutamate systems all contribute to the final perceived stimulus. The resemblance between the nicotine discriminative stimulus and those of the psychomotor stimulant drugs amphetamine and cocaine contributes to defining the nature of the addictive properties of nicotine. It is particularly interesting that acute and chronic exposure to caffeine produce quantitative and qualitative changes in the characteristics of the nicotine stimulus. Interactions of nicotine with caffeine and cannabinoids strengthen proposals that the use of one substance serves as a "gateway" in sequential shifts of the target substance for drug-seeking behaviour, with profound implications for the human use of the substances concerned.

Drug discrimination is also an important standard technique used in assessments of the abuse liability of novel psychoactive compounds, with relevance to attempts to develop novel nicotinic agonists for use as cognitive enhancers.

Abbreviations

5-HT	5-Hydroxytryptamine (serotonin)
7-OH-DPAT	(±)-7-Hydroxy-2-dipropylaminotetralin
ABT-089	2-Methyl-3-(2-(S)-pyrrolidinylmethoxy)pyridine
ABT418	(*S*)-3-Methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole
BP897	1-(4-(2-Naphthoylamino)butyl)-4-(2-methoxyphenyl)-1A-piperazine
CGS 10746B	(5-(4-Methyl-1-piperazinyl)imidazo[2,1-*b*][1,3,5]-benzothiadiazepine
DOI	(±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane
DOB	1-(4-Bromo-2, 5-dimethoxyphenyl)-2-aminopropane
GBR-12909	1-[2-[Bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl] piperazine
GTS-21	3-(2,4-Dimethoxybenzylidene)-anabaseine
ICS-205930	[(1S,5S)-8-Methyl-8-azabicyclo[3.2.1]octan-3-yl]1H-indole-3-carboxylate
MDL 72,222	8-Methyl-8-azabicyclo[3,2,1]octan-3-yl 3,5-dichlorobenzoate

MK 212	6-Chloro-2-(1-piperazinyl)pyrazine
MPEP	2-Methyl-6-(phenylethynyl)pyridine
MS-245	5-Methoxy-(N_1-benzenesulfonyl)-*N*,*N*-dimethyltryptamine)
NPA	R(−)-10,11-dihydroxy-*N*-*n*-propylnoraporphine
PD 128,907	S(+)-(4aR,10bR)-3,4,4a,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol
Ro-60–0175	(S)-2-(6-chloro-5-fluoro-indol-1-yl)-1-methylethylamine
SCH23390	(R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol
SKF38393	1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol
SKF81297	6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SKF82958	(±)-6-Chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SKF82958	3-Allyl-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SR 144528	*N*-[(1S)-Endo-1,3,3-trimethylbicyclo-[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
SSR591813	(5aS,8S,10aR)-5a,6,9,10-tetrahydro,7H,11H-8,10a-ethanopyrido[2',3':5,6]pyrano[2,3-d]azepine
TC2559	((*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine
U-101,387	4-[4-(2-Isochroman-1-ylethyl)piperazin-1-yl] benzenesulfonamide
URB 597	Cyclohexyl carbamic acid 3'-carbamoyl-biphenil-3-yl-ester
WO 03/062224	(1-Methyl-4-(2-chloro-4-hydroxyphenylthio)-piperidine
WO 01/60821A1	(R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide)

1 Introduction

Drug discrimination methodology provides an approach for objective, quantitative study of the perception of psychoactive drug effects that can be applied to substances across numerous pharmacological classes in either human or animal subjects. It is therefore no surprise that many drug discrimination studies have been conducted with nicotine and related ligands for its receptors; more remarkable is the fact that several of the first studies in the entire drug discrimination field were built around nicotine. The pioneering investigation by Morrison and Stephenson (1969) was not only the first known study of nicotine discrimination but it was also one of the very first reports of the discrimination of any drug using the two-lever operant conditioning techniques that subsequently became ubiquitous within the field. Ironically, this report emerged from the laboratories sponsored by the tobacco industry in Britain not long before their closure and at a time when other tobacco firms denied that

nicotine should be considered as a drug. By providing a robust bioassay system for some CNS actions of nicotine, drug discrimination has been particularly useful for investigating its receptor targets and their interactions with diverse neurotransmitter systems.

In this review we aim to provide a systematic summary of progress with respect to behavioural and pharmacological factors that modulate the nicotine discriminative stimulus (Sect. 3 below), its origin in terms of brain regions and nicotinic receptor subtypes (Sects. 4 and 5), and its secondary pharmacological mediation via dopamine and other pathways (Sect. 6). Studies with ethanol are not reviewed although it weakly and rather inconsistently attenuates nicotine discrimination (e.g. Le Foll and Goldberg 2005); such work does not shed light on the mode of action of nicotine because ethanol acts through several classes of receptor. Drug discrimination is also an important technique used in assessments of the abuse liability of novel psychoactive compounds (Ator and Griffiths 2003) for which purpose it is recommended by both the U.S. *Food and Drug Administration* and the *European Medicines Agency*. For example, it may be used in attempts to assessments of novel nicotinic agonists under development as cognitive enhancers. The partial substitution of abused psychomotor stimulant drugs such as amphetamine and cocaine for nicotine contributes to the evidence that nicotine is itself a drug of dependence and such studies are reviewed below in relation to the contribution of dopamine to nicotine discrimination. Nicotine has also been used as a prototypical psychoactive substance for studies of some general principles underlying drug discrimination behaviour and work of this type is also reviewed (Sect. 3.4).

Publications for inclusion in the review have been identified primarily by searches of the drug discrimination database (www.dd-database.org). Such a search in November 2007 yielded a total of 262 publications in which subjects were trained to discriminate nicotine; 144 were full-length reports of original investigations, the remainder being comprised of abstracts, reviews and book chapters. The great majority of studies used operant conditioning techniques although 14 of the earlier reports, in the years 1971–1982, relied upon mazes. Rats were the subjects in 125 of the full reports, the rest being comprised of studies in monkeys (2), mice (5) and humans (12). Only eleven papers used direct manipulations in the brain such as intra-cerebral drug injections or lesions, and a further two studies used genetically modified (knockout) mice. Other studies included investigations of psychological phenomena such as associative blocking, overshadowing, drug trace discrimination and occasion setting that often used nicotine as one element in compound cues generated by drug mixtures (20 reports). A further 79 papers included tests with nicotinic agonists and antagonists in subjects trained to discriminate non-nicotinic compounds and these studies are included in the review when they shed light on nicotine's mechanisms of action. The main previous reviews of nicotine discrimination have included those of Rosecrans et al. (1978), Stolerman (1987), Di Chiara (2000) and Le Foll and Goldberg (2006). The present article does not aim to be fully comprehensive but rather focuses on the studies most relevant to the research questions that it addresses.

2 The Basis of Drug Discrimination

All known drugs of abuse generate an internal stimulus (cue) that can be recognised, but the ability of a compound to provide discriminative stimuli is not a phenomenon that is exclusively related to abuse potential. Any compound that produces a reliable internal cue upon administration can be used to support discrimination training. In the most basic assessment of discriminative stimulus effects, in *drug training sessions* the subject is given nicotine and then is required to respond in a particular way (e.g. by pressing a lever or choosing an arm of a maze to obtain food reinforcers), thereby indicating that they recognise that they have received drug. To confirm that they recognise the specific internal stimulus produced and not the effect of drug administration itself, they are also given placebo administrations (*non-drug training sessions*) and are required to respond in a different way to indicate that they recognise a different internal state. There must not be any differences between exteroceptive stimuli present during drug and vehicle training sessions that could be confounded with interoceptive (drug) effects. Once trained, the subjects can be used in tests of stimulus generalisation which assess the ability of different compounds to mimic or to block the internal cue generated by the training drug (which is usually nicotine in the studies considered here).

The first recorded example of nicotine discrimination was the study of Morrison and Stephenson (1969) where Lister hooded rats were trained to make different behavioural responses depending on whether or not they had been injected with nicotine or saline. Rats learned to associate a 0.1 ml water reward with pressing one lever following nicotine (0.2 mg kg^{-1}) administration[1], whereas pressing another lever resulted in an electric shock. On other days, animals learned to press the saline-associated lever following saline injections and any responses on the "nicotine correct" bar were punished. Only the first response of each session was considered when scoring the rat's choice, but the rat was free to respond throughout the entire 10 min session. Figure 1, drawn from the data of Morrison and Stephenson (1969), shows the results of a dose–response determination with nicotine and evidence from tests with mecamylamine and chlorisondamine that nicotine's interoceptive cue was mediated through central nicotinic receptors. These observations were confirmed and extended in later work demonstrating that the nicotine cue exhibited classical pharmacological features with respect to time–course of action and dose–response characteristics that correlated appropriately with plasma and brain nicotine concentrations (Rosecrans and Chance 1977, 1978; Pratt et al. 1983). The nicotine cue was antagonised by mecamylamine, a nicotinic receptor antagonist that acts both centrally and peripherally, but not by the peripherally acting antagonist, hexamethonium, consistent with the earlier findings (Morrison and Stephenson 1969). Receptor specificity was also shown by the fact that the muscarinic antagonist atropine was unable to block the nicotine cue. The drug discrimination assay was sensitive to the

[1] The mg/kg doses of nicotine specified here and subsequently are those of nicotine base.

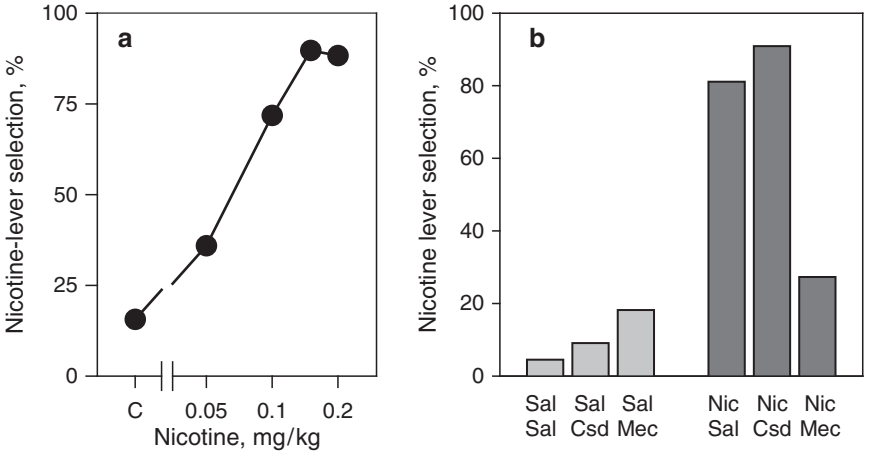

Fig. 1 (**a**) Dose–response curve from rats trained to discriminate between 0.2 mg kg^{-1} of nicotine and saline. Data are from 13 rats, each tested at least three times at each dose. (**b**) Block of nicotine (0.2 mg kg^{-1}) discrimination by the centrally active nicotinic antagonist mecamylamine (0.25 mg kg^{-1}) but not by chlorisondamine at a 0.025 mg kg^{-1} dose that blocks peripheral nicotinic cholinoceptors but does not penetrate into the CNS (Sal, Saline; Nic, Nicotine; Csd, Chlorisondamine; Mec, Mecamylamine). Chlorisondamine blocks nicotine discrimination when injected intra-cerebroventricularly (Kumar et al. 1987, data not shown). Data shown are for the number of sessions that began with selection of the nicotine-appropriate lever expressed as a percentage of the total number of sessions. Redrawn with permission from Tables 1 and 2 in Morrison CF, Stephenson JA. Nicotine injections as the conditioned stimulus in discrimination learning (1969), Copyright Springer

differences between the stereoisomers of nicotine and the (+)-isomer was found to possess about one-tenth of the potency of the (−)-isomer (Meltzer et al. 1980; Goldberg et al. 1989).

Thus drug discrimination can serve as an in vivo surrogate assay for receptor activation where the cue is selective for a particular receptor. It is a selective and sensitive in vivo measure of neuronal and receptor mechanisms and can help researchers to understand more of the brain's fundamental processes. It is the only assay allowing a direct test in which subjects that do not have language (e.g. rodents) can detect the presence of a psychoactive substance in the body and define the extent of the similarity of its effects to those of other substances.

3 Factors that Modulate Nicotine Discrimination

3.1 Age, Sex, Strain of Subjects

Nicotine discrimination studies have been conducted in humans, squirrel monkeys, rats and mice. However only in humans as there been any systematic approach to examine whether there are sex differences in discriminative effects. Female smokers

are less able than male smokers to detect the presence or absence of a nicotine stimulus after a nasal spray, suggesting a reduced sensitivity to nicotine in females (Perkins et al. 1994) and have been reported in general to be less sensitive to the discriminative stimulus effects of nicotine, particularly at lower doses (Perkins 1999). In a study examining the role of acute tolerance in the perception of the discriminative stimulus, nicotine pre-treatment through skin patches attenuated nicotine-appropriate responding during generalisation tests, but only in women, (Perkins et al. 2001). These and other studies in human subjects are reviewed in more depth elsewhere in this book (see chapter "Discriminative Stimulus Effects of Nicotine in Humans" in this volume).

In contrast, some animal studies have suggested that female rats have an increased sensitivity to nicotine although these have assessed acute antinociceptive effects and decreases in motor activity (e.g. Cronan et al. 1985; Craft and Milholland 1998) or chronic increases in locomotion (Kanýt et al. 1999). However, female mice of three strains were reported to be less sensitive than males to motor depressant effects of nicotine (Hatchell and Collins 1977). Gonadal hormones have been suggested to play a part in stimulus control as responses to nicotine during ethanol withdrawal were lower in intact females compared with ovariectomised females and males (Jung et al. 2000).

There are no studies in any species examining whether age affects the discrimination of nicotine. In humans, ethical concerns restricts the ability to undertake these studies in children and in animals, the length of time it takes to train an animal to discriminate between drug and vehicle precludes testing in very young animals. There are also no studies looking at whether old age alters the perception of nicotine, where one might expect pharmacokinetic changes to have an impact.

Tests for strain differences in nicotine discrimination are also sparse. Many different strains have been used but it is not easy to make direct comparisons of relative sensitivities simply because the different methodologies used, with varying training doses and schedules of reinforcement, can affect the results. Where specific strains have been compared within a study, nicotine discriminative effects were not the primary outcome. For example, alcohol preferring (P) rats and alcohol non-preferring (NP) rats were trained to discriminate 1 g kg^{-1} ethanol and were then given nicotine at different doses. At 0.42 mg kg^{-1} of nicotine only the P rats showed a partial (~58%) generalisation to nicotine (Gordon et al. 1993). In rats trained to discriminate ethanol (0.5 g kg^{-1}), nicotine substituted at the level of 80% in P rats and 33% in NP rats. When trained to discriminate nicotine (0.21 mg kg^{-1}), ethanol did not substitute for nicotine in either strain, suggesting asymmetrical strain differences in discrimination (McMillan et al. 1999). Fischer-344 (F344) rats exhibited lower sensitivity to nicotine than either Sprague Dawley or Lewis rats (Rosecrans and Schechter 1972; Philibin et al. 2005). The role of pharmacokinetics was investigated in Sprague–Dawley rats, but their low sensitivity could not be attributed to reduced brain levels of nicotine (Rosecrans and Schechter 1972). Mice of both C57BL/6 and DBA/2 strains acquired the discrimination over about 40 sessions using a 0.8 mg kg^{-1} training dose and there were no clear differences between the strains, although the study was limited in scope (Stolerman et al. 1999 and Fig. 2b below).

3.2 Pharmacological Variables

Many researchers using drugs from other pharmacological classes have demonstrated that the training dose selected for discrimination training is critical in regulating the specificity of the resulting generalisation profiles. The role of training dose in determining ED_{50} values for nicotine is illustrated for rats and mice in Fig. 2. An analysis of the literature suggests that if one compares across nine studies where the same rat strain (Sprague–Dawley) was used, there is a correlation between training dose and ED_{50} values ($r^2 = 0.65$, $p < 0.01$); training with lower doses of nicotine seemed to reduce the ED_{50}. Although it is possible that this effect is a consequence of other differences in methodology a within-study comparison of three training doses of nicotine in Lister hooded rats using consistent methodology yielded entirely concordant results (Fig. 2a). Figure 2b shows that a similar correlation between training dose and ED_{50} was also demonstrable in mice.

Some studies have examined the time–course for onset and offset of the nicotine discriminative stimulus. The effects of varying pre-treatment time (PT) have most often been assessed by fixing times during training and then varying the pretreatment time in testing. Hirschhorn and Rosecrans (1974) examined two doses of nicotine (0.2 and 0.4 mg kg^{-1}) with a training PT of 5 min. The onset of the nicotine effect during testing was clearly apparent 5 min after the subcutaneous

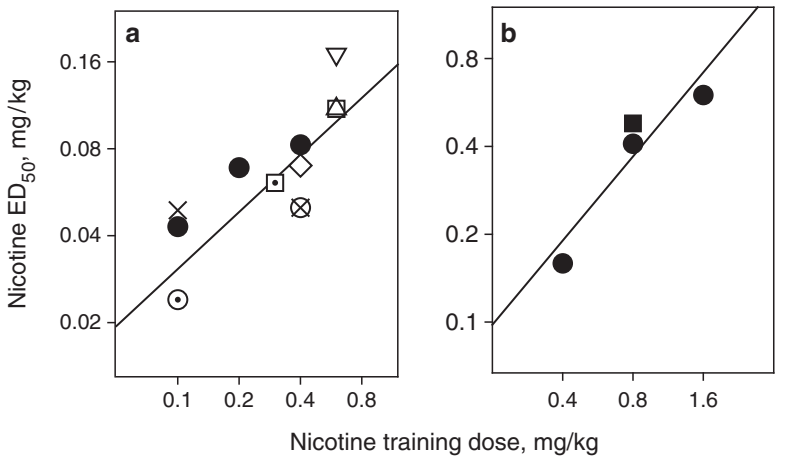

Fig. 2 (**a**) Rat ED_{50} values from diverse studies using various methodologies to show the relationship with the training dose of nicotine. Data for studies in Sprague Dawley rats are represented by open symbols (*open square with dark centre*, Batman et al. 2005; *open triangle*, Bondarev et al. 2003; *open circle with dark centre*, Desai et al. 2003; cross, Gasior et al. 1999; open diamond, Le Foll and Goldberg 2005; *open circle*, Mansbach et al. 2000; *open inverted triangle*, Young and Glennon 2002; *open square*, Young et al. 2006; $r^2 = 0.65$). Data from a study comparing three doses of nicotine in Lister hooded rats are shown by solid symbols (*filled circle*, Stolerman et al. (1984). (**b**) Mouse ED_{50} values from Stolerman et al. (1999) showing relationship ($r^2 = 0.80$) with the training dose in the C57BL/6 strain (*filled circle*) and the ED_{50} for DBA/2 mice at one training dose (*filled square*)

injections, but even at 60 min after dosing with 0.4 mg kg^{-1}, animals still showed partial generalisation to the training dose of nicotine, with weaker effects detectable for up to 120 min (Hirschhorn and Rosecrans 1974). With 0.2 mg kg^{-1}, selection of the nicotine lever was not as high even at the shortest PT, but animals still preferentially selected the nicotine lever until more than 80 min after dosing. In conjunction with the discrimination data, Hirschhorn and Rosecrans (1974) measured levels of nicotine in three brain areas and suggested that as long as the brain concentration was equal to or greater than 1 μMg^{-1} tissue, the animal was able to detect that it had received nicotine. Other studies supported these findings and indicated that plasma concentrations of nicotine were often within the same range as those for cigarette smokers who inhaled (Pratt et al. 1983).

A factorial design has been used to separate the influence of PT during training from that during testing (Stolerman and Garcha 1989). Different groups of rats were trained with PT of 5, 25 and 45 min and then dose–responses curves for nicotine were obtained in each group at all three PT. Increasing the PT during testing *increased* the ED_{50} from 0.062 to 0.171 mg kg^{-1}, as would be expected from the lower plasma concentrations of nicotine at longer PT. However, increasing the PT during training *decreased* the ED_{50} at the time of testing from 0.170 to 0.077 mg kg^{-1}; the lower plasma concentrations of nicotine at longer PT appeared to be functionally equivalent to training with lower doses of nicotine, a manipulation known to decrease ED_{50} values (see above). Thus, the effects of changing the PT during training and testing were similar in magnitude but opposite in direction, a finding that could be explained by pharmacokinetic considerations together with knowledge of the role of training dose in nicotine discrimination.

Craft and Howard (1988) examined the effect of the route for administering nicotine; rats were trained to discriminate nicotine (0.5 mg kg^{-1}) given orally (per os). Dose–response testing then found little difference between ED_{50} values for the subcutaneous, intra-peritoneal and oral routes (0.076, 0.090 and 0.073 mg kg^{-1} respectively). Administration transdermally by placing the nicotine solution on the rats' shaved dorsal skin also produced discriminable effects although the dose of nicotine needed was approximately an order of magnitude greater (ED_{50} = 1.34 mg kg^{-1}). In the same year a report on the use of nicotine patches in smoking cessation appeared (Buchkremer et al. 1988).

Tolerance and physical dependence are characteristic features of the repeated administration of many drugs including nicotine. Tolerance is defined by a rightward shift of the dose–effect curve, resulting in the need for increased doses of drug to produce the same magnitude of effect. Acute tolerance to the nicotine cue in rats has been documented in several studies (e.g. James et al. 1994; Robinson et al. 2006). Chronic tolerance to some behavioural effects of nicotine is observed with repeated injections and can persist for lengthy periods of time (e.g. Stolerman et al. 1973) but few similar studies with the nicotine discriminative stimulus have been published. Repeated injections of nicotine, either once or three times daily, with doses ranging from 0.1–1.2 mg kg^{-1}, did not affect nicotine discrimination (Shoaib et al. 1997). Continuous infusion with a large dose of nicotine (6.4 mg kg^{-1} per day) produced a small increase in the ED_{50} for nicotine, although the authors questioned the

adequacy of the chronic dosing regimen because no signs of nicotine withdrawal were manifest (Shoaib et al. 1997). The magnitude of tolerance to the nicotine discriminative stimulus appeared to be less than might have been expected from studies of tolerance in opiate or alcohol discrimination (Hiltunen and Järbe 1990; Sannerud and Young 1987; Witkin et al. 1982).

3.3 Behavioural Variables

Two main methodological approaches have been utilised for discrimination studies, involving operant chambers and T-mazes, each of which relies on establishing a conditional discrimination where subjects learn an association between a stimulus and an action–outcome relationship. This can be summarised as: if stimulus A is present make one response; if stimulus B is present make a different response. In the typical operant procedure, pressing one lever for reinforcement is associated with the prior administration of nicotine, and pressing a second lever is associated with vehicle administration (although there are studies where three or more manipulanda are used). Responses on the inappropriate lever are never reinforced in training and may reset the response requirement. Typically, the schedule of reinforcement is the same for the drug as for the vehicle responses although investigations of response bias have differentially manipulated the contingencies for the two manipulanda (Koek and Slangen 1982; McMillan and Wenger 1984).

Evidence shows that the schedule of reinforcement is a crucial determinant of the type of data obtained with the drug discrimination approach (e.g. Stolerman 1991; McMillan et al. 2001). If responding is maintained by a variable interval schedule (i.e. subjects receive a pellet for the first response made after an unpredictable period of time) drugs produce graded generalisation curves allowing an estimate in an individual animal of how closely the effects of a test compound resemble those of the training drug. This type of approach allows discrimination tests to be conducted in extinction as the unpredictable time for reinforcement makes it difficult for subjects to distinguish between training sessions and extinction tests. On the other hand, with fixed ratio schedules stimulus control exerted by drug states is stronger, but dose response curves are predominantly quantal in nature (i.e. drug effects are of an "all or none" nature); thus, in any given test session almost 100% of responses occur on one lever and almost 0% of responses on the other lever. This approach allows determination of intermediate levels of generalisation only from a population response (i.e. if four of ten subjects press the drug lever, the drug lever-selection score is 40%).

The possible effects of different types and magnitudes of reinforcer on nicotine discrimination have not been studied directly although several types have been used. Typically 45 mg food pellets are used for rats and 25 mg pellets for mice although some groups have used sweetened milk as a liquid reinforcer. However, water reinforcers following restricted access to water could also support nicotine discrimination learning (e.g. Morrison and Stephenson 1969; Sanchez et al. 1998; Zaniewska et al. 2006). Varying restricted access to food has not been shown to

affect the discriminative stimulus effects of other drugs. For example, the discriminative stimulus effects of morphine were not modified consistently in rats maintained at reduced body weights by restricted feeding in a study where the animals pressed the appropriate levers to avoid foot shock (Ukai and Holtzman 1988) and food restriction also failed to modify the discriminative stimulus effects of pentobarbitone or phencyclidine in pigeons responding under fixed interval and second-order schedules of food reinforcement (Massey and McMillan 1987; Li et al. 1995).

More rarely, navigation of a T-maze has been used for drug discrimination studies. Early studies used a T-maze procedure where rats were trained to select one of the two arms when nicotine was administered, whereas entries into the opposite arm were punished by foot-shock (Romano et al. 1981; Schechter and Rosecrans 1971a, b; Overton 1982). Nicotine proved to have moderate relative discriminability, as measured by the sessions to criterion performance (20 sessions at 0.55 mg kg^{-1}; Overton 1982). More recently, a food-rewarded discrimination procedure using a T-maze has been described, but no studies involving nicotine have been identified (Colombo et al. 1996).

3.4 Behavioural Mechanisms for Drug–Drug Interactions

In studies of drug mixture discriminations where nicotine served as one element of a two-component stimulus, the main characteristics of the discrimination were similar to those seen for several other binary mixtures. For example, rats were trained to discriminate a mixture of nicotine and the short-acting benzodiazepine midazolam, two dissimilar psychoactive drugs that did not exhibit appreciable cross-generalisation. Figure 3a shows that in generalisation tests each drug alone engendered very considerable amounts of mixture-appropriate responding (Stolerman et al. 1987). Thus, rats trained to discriminate mixtures of these drugs identified and responded to their component drugs. Similar results were obtained with other drug mixtures involving substances from several different pharmacological classes (Stolerman et al. 1991). The relevance of this research to interpretations of studies on a possible dopamine-mediated element in the nicotine discriminative stimulus is explained in Sect. 6.1 below.

Interactions between the component drugs in mixtures were observed that could be explained by psychological rather than by pharmacodynamic or pharmacokinetic mechanisms. Overshadowing is shown by a weakening of conditioning to a normally adequate stimulus by conditioning it in compound with different, more salient stimulus (Mackintosh 1974). When conditioning was carried out with a mixture of nicotine and relatively large doses of midazolam (Fig. 3b), very little stimulus control accrued to the nicotine component despite the use of doses of nicotine that were well within the discriminable range when used alone (Stolerman et al. 1987; Garcha and Stolerman 1989). Midazolam did not function as a pharmacological antagonist of nicotine because it did not attenuate a simple nicotine discrimination. Similar results were seen with several other drug mixtures and it was suggested

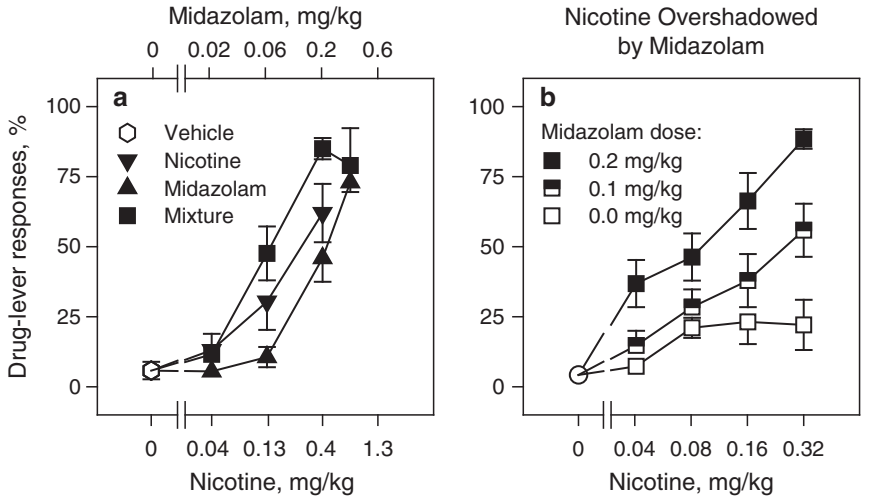

Fig. 3 (**a**) Rats were trained to discriminate a mixture of nicotine (0.4 mg kg^{-1}) and midazolam (0.2 mg kg^{-1}) from saline ($n = 8$). Each component of the mixture administered alone increased drug-appropriate responding in a dose-related manner and their maximal effects were close to those of the drug mixture. Results shown as mean percentage of responses on the drug-appropriate lever (± s.e.m.) during 5-min sessions when no food was available (redrawn from Stolerman et al. 1987, Discriminative stimulus effects of a nicotine–midazolam mixture in rats). (**b**) Discriminative effects of nicotine in three groups of rats trained to discriminate nicotine (0.32 mg kg^{-1}) only or mixtures of the same dose of nicotine with the doses shown of midazolam ($n = 8$). Midazolam overshadowed the discriminative effect of nicotine in a dose-related manner. Redrawn from Mariathasan and Stolerman (1993). Overshadowing of nicotine discrimination in rats: a model for behavioural mechanisms of drug interactions?

that overshadowing of one drug by another was the common underlying mechanism (Garcha and Stolerman 1989; Mariathasan et al. 1991; Mariathasan and Stolerman 1993a; White and Stolerman 1996). The response to nicotine could be restored by extinguishing the response to the other drug (White and Stolerman 1996). Associative blocking was shown when a previous history of training to discriminate nicotine prevented conditioning to a second drug (midazolam) during subsequent discrimination training with a mixture of the two substances (Stolerman and White 1996). Thus, the characteristics of discriminations based on drug mixtures that contained nicotine or other substances were in accordance with predictions derived from studies with compound exteroceptive stimuli (Mackintosh 1974).

Further experiments aimed to determine whether stimulus control could be established when the effects of nicotine were present prior to training sessions but not during the sessions. Thus, rats were first exposed to nicotine and then its effects during training sessions were blocked by administering a nicotine antagonist before training; under these conditions, drug discrimination developed slowly and with low asymptotic accuracy (Stolerman et al. 2002). The finding was interpreted

in terms of stimulus control by the pre-session effects of nicotine although it was clear that effects of nicotine present during sessions were much more effective as discriminative stimuli.

Later studies tested whether a second drug could serve as a mediating stimulus that increased the strength of stimulus control by such pre-session effects of nicotine. In these studies, injections of either nicotine or saline were followed after 5 min by administration of midazolam as a putative mediating stimulus. The nicotine antagonist mecamylamine was then administered in order to block effects of nicotine during training sessions. Midazolam did not facilitate the acquisition or magnitude of nicotine-induced stimulus control but extinction tests revealed the pivotal finding of the study; stimulus control by nicotine was detected in the presence of midazolam but not in its absence (Stolerman and Mariathasan 2003). The results implied that the discriminative stimulus effects of one drug could be mediated by the action of a second substance. This finding was conceptualised in terms of occasion setting (Holland 1991), with nicotine serving as the feature and midazolam as the target stimulus. More recent studies have used Pavlovian paradigms to show occasion setting with nicotine (Palmatier et al. 2004, 2005), thus confirming that nicotine could serve as a feature stimulus in occasion setting by means of procedures very different from those of Stolerman and Mariathasan (2003). In generalisation tests, partial substitution was evident with amphetamine, and mecamylamine dose-dependently blocked nicotine's control of the conditioned response whereas hexamethonium had no effect (Palmatier et al. 2004, 2005). Therefore the pharmacological characteristics of the nicotine response in the Pavlovian procedure have to date resembled those in operant drug discrimination paradigms.

4 Neuroanatomical Origin of Nicotine Discriminations

4.1 Role of the Central Nervous System

The nicotine discriminative stimulus originates primarily in the central nervous system. When nicotine is given systemically, it can be discriminated with high levels of behavioural specificity. Centrally acting agonists such as nornicotine fully substituted for nicotine whereas the peripherally acting nicotinic agonist methylcarbamylcholine when given systemically did not (Desai et al. 1999). Nicotinic antagonists such as dihydro-β-erythroidine and mecamylamine antagonise the nicotine discriminative stimulus. Contrastingly, antagonists such as chlorisondamine or pentolinium when given systemically had no effect but when administered by the intracerebroventricular route they blocked nicotine discrimination (Kumar et al. 1987). These are molecules that penetrate the blood brain barrier poorly due to the presence of quaternary ammonium (NH_4^+) ions, although the quaternary antagonist hexamethonium surprisingly failed to block nicotine even when administered into a lateral cerebral ventricle.

4.2 Role of Brain Regions

A small number of studies have examined the effects of locally administered nicotine in animals trained to discriminate systemically administered nicotine. Nicotine administered into the lateral ventricle substituted for nicotine (Chance et al. 1978; Miyata et al. 2002; Schechter 1973). Meltzer and Rosecrans (1981) reported that the hippocampus and medial reticular formation contributed to the mediation of the cueing properties of nicotine. Administration of 8 μg doses of nicotine into the dorsal hippocampus resulted in significant partial generalisation in two studies (Meltzer and Rosecrans 1981; Shoaib and Stolerman 1996) whereas a similar trend was not significant in a third study where animals were trained with different doses of nicotine and schedules of reinforcement (Miyata et al. 2002). Local infusions of 40 μg of nicotine in the medial prefrontal cortex resulted in complete substitution but there was only partial substitution in the ventral tegmental area (Miyata et al. 2002). There is contradictory evidence for the involvement of the nucleus accumbens with Shoaib and Stolerman (1996) reporting no generalisation at 2–8 μg whereas Miyata et al. (2002) reported almost full generalisation albeit at much higher doses that were twice those required to produce a greater substitution when given directly into the medial prefrontal cortex. Thus, the evidence strongly supports a central site of action for the discriminative stimulus of nicotine and this is likely to be located in the mesocorticolimbic dopaminergic neurons; although rather few brain regions have been investigated, the medial prefrontal cortex seems to be primarily involved, with other areas tested so far appearing to be of secondary importance.

5 Primary Pharmacological Origins

5.1 Role of Nicotinic–Cholinergic Receptors

Acetylcholine released from nerve terminals binds with two types of receptors, the nicotinic receptors which are members of the ligand-gated ion channel superfamily and muscarinic receptors which are G-protein coupled receptors. Early evidence showed that the nicotinic discriminative cue was generated by the interaction of nicotine at nicotinic receptors and not at muscarinic receptors. Muscarinic agonists such as oxotremorine and arecoline did not substitute for nicotine (Pratt et al. 1983; Wiley et al. 1996) nor did antagonists such as atropine block the nicotine stimulus (Schechter and Rosecrans 1971a; Wiley et al. 1996). Additional studies have also concluded that muscarinic antagonists such as atropine do not generalise to the nicotine stimulus cue (Pratt et al. 1983). Acetylcholinesterase inhibitors such as physostigmine neither substitute for nor block the nicotine stimulus (Pratt et al. 1983; Wiley et al. 1996). This lack of generalisation is symmetrical as nicotine does not substitute for arecoline or scopolamine (Overton 1977; Wiley et al. 1996). The conclusion is that the nicotine discriminative stimulus is

mediated predominantly at nicotinic rather than muscarinic receptors. The lack of generalisation with acetylcholinesterase inhibitors may be attributed to their non-selective effects on acetylcholine concentrations at both types of cholinoceptors.

5.2 Nicotinic Receptor Subtypes

The early characterisation of neuronal nicotinic acetylcholine receptors (nAChR) was based on binding assays with nicotinic radioligands (e.g. Bencherif et al. 1995; Clarke et al. 1984). The pharmacological heterogeneity of nAChRs revealed by these studies was extended by the cloning of a family of genes encoding various subunits (Le Novère et al. 2002; Lindström et al. 1998). Twelve genes coding for nAChR subunits have been cloned and are divided into two families of nine α subunits (α2–α10) and three β subunits (β2–β4). Pharmacologically active receptors contain five subunits (comprising either homo- or hetero-pentamers) surrounding a pore through which cations, predominantly Na+ and K+, pass. Both α and β subunits are thought to contribute towards the pharmacological specificity of nAChR subtypes (Luetje and Patrick 1991). Receptor subunit composition is covered in more detail in the chapter "Molecular targets of nicotine action in the brain". The predominant receptor subunit compositions in rodent brain are α4β2-containing, but specific brain regions also contain significant α3β4-containing receptors and α7 homo-pentameric receptors.

5.3 Generalisation Tests with Nicotinic Agonists

The predominant alkaloid found in tobacco is (−)-nicotine; other molecules found in tissues of smokers are either present in tobacco smoke or are metabolites of nicotine, including (+)-nicotine, (+)-nornicotine, (−)-nornicotine and (−)-cotinine, the major metabolite (Clark et al. 1965). In animals trained to recognise the stimulus produced by (−)-nicotine, (+)-nicotine fully substituted for (−)-nicotine but was about one-tenth as potent. There was no stereoselectivity in responding observed with the metabolite nornicotine and both (+) and (−)-isomers fully substituted for nicotine but again were 10-fold less potent. (−)-Cotinine also substituted for nicotine at very high doses but this could be explained by the presence of small amounts of (−)-nicotine in the sample of cotinine (Goldberg et al. 1989).

Ligands selective for various nicotinic receptor subtypes have been used to understand the pharmacological specificity of the interoceptive stimulus cue. Ligands with high affinity for the α4β2-containing receptors have been assessed in animals trained to discriminate nicotine from vehicle. Those agonists that are selective for α4β2-containing receptors show high levels of substitution for nicotine with little or no effect on response rates at doses that fully generalise to the nicotine stimulus cue (Smith et al. 2007). In contrast, agonists that have high affinity for α4β2-containing receptors but also have significant activity at other receptors including

α3β4-containing receptors show generalisation to the nicotine cue but may also have other actions such as reduction of response rates. Nicotine, ABT594, A85380 and 5-iodo-A85380 all show high affinity for the α4β2-containing receptors but also have activity on α3β4 receptors (all Ki (α3β4) $< 1\,\mu M$; Smith et al. 2007). TC2559 has high affinity for and acts selectively at the α4β2-containing receptors (Ki $= 22$ nM), with no measurable affinity at α3β4 or α7 containing receptors. As shown in Fig. 4, these compounds show complete generalisation to the nicotine stimulus cue with

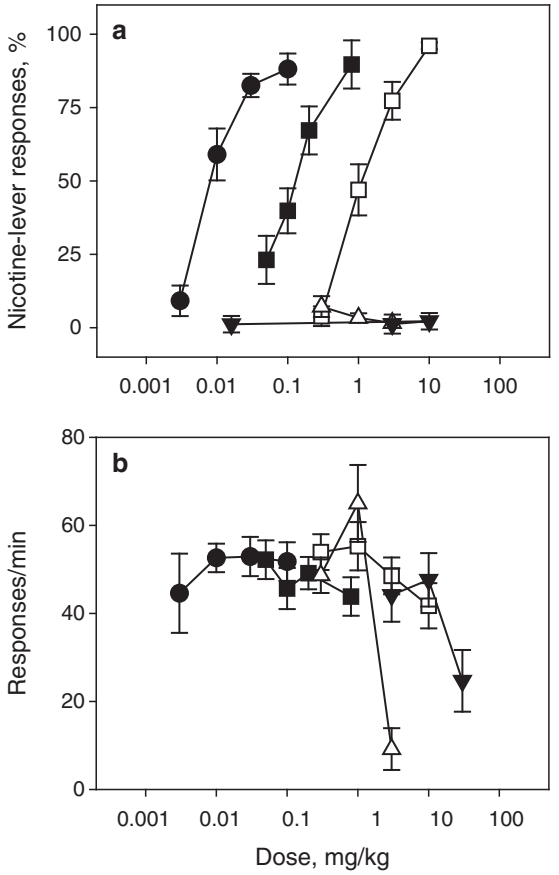

Fig. 4 Discriminative stimulus (**a**) and response rate (**b**) effects of A85380 (*filled circle*), TC2559 (*open square*), nicotine (*filled square*);WO 03/062224 (*open triangle*) and WO 01/60821A1 (*filled triangle*) in rats trained to discriminate nicotine (0.4 mg kg^{-1}) from 5% glucose. All results are presented as means ± s.e.m. for 15 min tests where responses on both levers were reinforced on independent tandem VI 30 s FR10 schedules. (**a**) shows results for the percentage drug-appropriate responses following administration of each of the compounds and (**b**) shows the overall response rates per minute. All experiments were conducted between-subjects with group sizes of 6–16. Redrawn with permission from Figs. 1, 5 and 6 in Smith et al. (2007). Ligands selective for alpha4beta2 but not alpha3beta4 or alpha7 nicotinic receptors generalise to the nicotine discriminative stimulus in the rat. Copyright Springer

no effects on response rates at the highest doses tested in the drug discrimination studies (Smith et al. 2007; Zaniewska et al. 2006).

The nicotinic partial agonists varenicline and cytisine also have high affinity for α4β2-containing receptors with measurable activity at α3β4 and in the case of varenicline activity at α7 receptors as well (Rollema et al. 2007; Smith et al. 2007). Cytisine has produced partial generalisation in all studies reported. Varenicline partially generalised to the nicotine stimulus when using quantitative measurements (Smith et al. 2007) and fully generalised when the quantal approach was used (Rollema et al. 2007); both studies showed that striatal dopamine release was only ~60–70% of that with 10 μM nicotine. When higher doses were used to increase the level of substitution produced by these compounds, response rates were decreased (Smith et al. 2007). SSR591813, ABT − 089 and ABT418 are partial agonists with high affinity for α4β2 containing receptors. These molecules have also been shown to partially generalise to the nicotine discriminative stimulus and SSR591813 decreased response rates as the dose was increased (Brioni et al. 1995, 1997; Cohen et al. 2003).

In contrast, ligands selective for α3β4 receptors (WO 03/062224; Ki = 1.5 nM on α3β4 and 413.4 nM on α4β2 but no measurable activity on α7 receptors), or α7-containing receptors (for example WO 01/60821A1; Ki of 1.2 nM on α7 receptors but no measurable activity on either α3β4 and α4β2–containing receptors) did not generalise to the nicotine discriminative stimulus cue even at doses that reduced response rates (Smith et al. 2007). GTS − 21, a weak partial agonist of the α7 receptor also did not generalise to the nicotine discriminative stimulus (Briggs et al. 1997; Van Haaren et al. 1999).

Lobeline has been used in smoking cessation remedies and has been shown to displace nicotine from its binding sites with a Ki of 4 nM, but its pharmacological effects are not typically mediated through α4β2 receptors (Damaj et al. 1997). In drug discrimination, lobeline did not generalise with or block nicotine (Brioni et al. 1994; Reavill et al. 1990).

In conclusion, studies with nicotinic agonists are compatible with the view that α4β2-containing receptors play a critical and possibly unique role in generating the discriminative stimulus properties of nicotine.

5.4 Training with Other Nicotinic Agonists

Limited studies have been conducted where nicotinic agonists other than nicotine have been used for discrimination training. Rats trained with 1.9 or 6.2 μmol kg^{-1} ABT-418 were not able to discriminate ABT-418 from a saline solution following 50 days of training. In rats trained to discriminate 0.3 mg kg^{-1} nicotine, ABT-418 partially substituted at 1.9 and 6.2 μmol kg^{-1}. Even after 64 days training, ABT-089 at both 19 and 62 μM kg^{-1} did not result in discrimination whereas in animals trained to discriminate nicotine from saline, ABT-089 partially generalised to the nicotine cue at 62 μM kg^{-1} (Brioni et al. 1997). Cytisine (3 mg kg^{-1}) did sustain a discrimination which was learned within 50 sessions and nicotine fully generalised

in these animals, whereas in animals trained to discriminate nicotine, cytisine only partially generalised to the nicotine stimulus cue (Chandler and Stolerman 1997). Animals were not able to learn to discriminate WO 03/062224, a nicotinic agonist selective for the α3β4 receptors, from vehicle following 47 days training (Smith et al. 2007). As noted above, this compound also did not substitute for nicotine in trained animals, but was present in brain at concentrations greater than 2 μM at the training dose used.

5.5 Studies with Nicotinic Antagonists and Partial Agonists

Dihydro-β-erythroidine and erosydine have been characterised as competitive nicotinic antagonists that bind with high affinity to sites labelled with [^{3}H]-cytisine and [^{3}H]-nicotine. Neither compound binds with high affinity to α7 or neuromuscular junction (α1β1γδ) receptors (Decker et al. 1995) or seems to block the nicotinic ion channel as their binding is not displaced by mecamylamine (Williams and Robinson 1984). Both compounds have been shown to produce a dose-dependent block of nicotine discrimination that can be reversed by increasing the dose of nicotine. Thus, they shift the dose–response curve for the discriminative effect of nicotine to the right while not affecting response rates at doses that completely prevent animals from recognizing nicotine (Stolerman et al. 1997; Mansbach et al. 2000).

Systemically administered mecamylamine was first shown to antagonise the discriminative effects of nicotine in studies by Morrison and Stephenson (1969) and Hirschhorn and Rosecrans (1973, 1974). Mecamylamine is non-selective non-competitive antagonist that penetrates into the brain and is thought to block the ion-channel (e.g. Varanda et al. 1985). Hexamethonium, pentolinium and chlorisondamine are quaternary ganglion blockers that penetrate the blood–brain barrier poorly. Chlorisondamine completely blocked the discriminative effect of nicotine in tests when given 7 days after implantation of intra-cerebroventricular cannulae and this block persisted, albeit incompletely, for a further 21 days (Kumar et al. 1987). Pentolinium had a modest effect on nicotine discrimination when first given, and the effect did not persist. When hexamethonium was given centrally, it did not appear to modify either nicotine or cytisine discrimination (Kumar et al. 1987; Stolerman et al. 1983).

Antagonists with some selectivity for α7-containing receptors also exist, the best characterised of which is methyllycaconitine (MLA). Administration of MLA through both intra-cerebroventricular or systemic routes neither substituted for, nor antagonised, the nicotine cue in rats or mice (Brioni et al. 1996; Gommans et al. 2000). Dextromethorphan and dextrorphan appear to block nicotinic α3β4-containing receptors, but did not attenuate nicotine discrimination in rats (Wright et al. 2006). The preceding results suggest that nicotine's interoceptive stimulus originates mainly at heteromeric α4β2 receptors and confirm that the homomeric α7 receptors do not appear to play a major role.

Cytisine and varenicline are partial agonists with activity at the α4β2 binding site. In tests of nicotine discrimination, pretreatment with cytisine has no

appreciable effect on the discriminative stimulus properties of nicotine while significantly reducing the lever-press response rates (Reavill et al. 1990). Varenicline was reported by Le Foll and Goldberg (2006) to block the discriminative stimulus effects of nicotine, but subsequent publications have only reported full or partial substitution to the nicotine cue and no blockade (Rollema et al. 2007; Smith et al. 2007). Varenicline has been recently licensed as a smoking cessation aid while cytisine has been marketed and widely used for that purpose over 40 years in central and eastern Europe (reviewed by Etter 2006). Varenicline and cytisine may have efficacy in smoking cessation by occupying sufficient receptors to produce some of the effects of nicotine, thereby reducing craving, while at the same time blocking further activation of the receptor by nicotine itself. However, the data from discrimination studies to date provide rather limited support for its proposed partial agonist in vivo activity in rats and much more extensive studies are needed to clarify varenicline's action in these procedures; the possibilities include its use as a training drug, tests of generalisation with other partial agonists such as cytisine (not necessarily limited to studies in rats), and investigation of interactions with dopaminergic mechanisms.

5.6 Training with Nicotinic Antagonists

A small number of studies have employed nicotine antagonists during discrimination training, in contrast to their more common use to modulate discrimination of nicotinic agonists or other substances. Using the number of trials to criterion as the main factor, Overton attempted to quantify the relative discriminability of nicotinic agonists and antagonists using a T-maze procedure. Animals were able to discriminate mecamylamine, albeit at heroic doses of 10–30 mg kg^{-1} (Overton 1982). Garcha and Stolerman (1993) subsequently demonstrated that mecamylamine could sustain stimulus control in an operant conditioning procedure at a dose of 3.5 mg kg^{-1} which was still large in comparison with typical doses for blocking nicotine. Furthermore, nicotine did not generalise with or antagonise the mecamylamine stimulus, suggesting either that the mecamylamine and nicotine stimuli were not generated at the same central sites or that the non-competitive nature of mecamylamine's activity at nicotinic receptors prevented reversal by nicotine (Varanda et al. 1985). Some compounds known to penetrate poorly into the brain generalised (pentolinium) or partially generalised (hexamethonium) to the mecamylamine cue, whereas chlorisondamine did not generalise at the doses tested (Garcha and Stolerman 1993).

Studies comparing the effect of training with nicotine alone with nicotine plus mecamylamine have been conducted. Discrimination of nicotine alone was acquired within 50 sessions with an ED_{50} of 0.082 mg kg^{-1}. In combination with nicotine, mecamylamine reduced accuracy during acquisition. In extinction tests in rats trained with nicotine plus 0.2 mg kg^{-1} mecamylamine, the ED_{50} for nicotine was lowered to 0.036 mg kg^{-1}, an effect resembling that of training at a smaller dose of nicotine. In rats trained with nicotine and higher doses of mecamylamine, nicotine did not acquire stimulus control over behaviour (Mariathasan and Stolerman 1993b).

Rats have also been trained to recognise the interoceptive cue produced by the administration of 3.0 mg kg^{-1} mecamylamine 15 min before testing, with nicotine administered 120 min before testing (referred to as nicotine → mecamylamine discrimination) from saline → saline administration. In generalisation tests, mecamylamine alone (6 mg kg^{-1}) substituted for the stimulus cue and pre-treatment with 1 mg kg^{-1} nicotine, 120 min before test shifted the dose–response curve to the left. Administration of the nicotinic partial agonist SSR591813 produced partial substitution to this cue (Cohen et al. 2003). At present it is not clear how the field would be advanced by further studies that entail training with nicotinic antagonists.

5.7 Use of Genetically Modified Mice

The β2 nAChR subunit was the first of the nicotinic receptors to be targeted in gene manipulation experiments. Mice lacking the β2 subunit did not show nicotine-elicited dopamine release and also show impairments in intra-venous nicotine self-administration experiments (Picciotto et al. 1998; Maskos et al. 2005). Shoaib et al. (2002) showed that this mutation blocked the acquisition of nicotine discrimination at typical training doses of 0.4–0.8 mg kg^{-1} and the usual dose–response relationship for nicotine discrimination was abolished (Fig. 5a); discrimination was only apparent when a very high training dose (1.6 mg kg^{-1}) was used and this discrimination was weak, which suggests that the animals may have been discriminating effects of nicotine that were unrelated to the β2 subunit. The α2β4 receptor was suggested as an alternative. Animals with the same mutation acquired morphine discrimination normally, suggesting that the ability of the animals to perform conditional discriminations in general was not impaired (Shoaib et al. 2002).

In contrast to the findings with β2 knockout mice, mice lacking the α7 receptor showed no deficits in acquisition at either 0.4 or 0.8 mg kg^{-1} training doses of nicotine and Fig. 5b shows that these animals exhibited normal nicotine dose–response curves (Stolerman et al. 2004). Both the β2 and α7 knockout mice showed the usual sensitivity to the rate suppressant effects of nicotine, suggesting that these effects were mediated through a different receptor subtype. Nicotine discrimination has not been assessed in α4 null mutants although these animals have shown increased mesostriatal dopamine levels in the absence of nicotine (Marubio et al. 2003). The most likely candidates were receptors containing α3β4 subunits, although nicotine discrimination was not assessed in mutants lacking either of these subunits. Salas et al. (2004) showed that both β4 null mutants and mice heterozygous for α3 were less sensitive to nicotine-induced locomotor depression and seizures. It was not possible to test α3 null mutants which suffer high rates of perinatal mortality (Xu et al. 1999), whereas β4 null mutants show no gross phenotypes. In conclusion, the results to date with genetically modified mice support the findings from studies with nicotinic agonists and antagonists (Sects. 5.2.2–5.2.4 above) to the effect that the nicotine discriminative stimulus most likely originates predominantly at receptors of the α4β2 subtype.

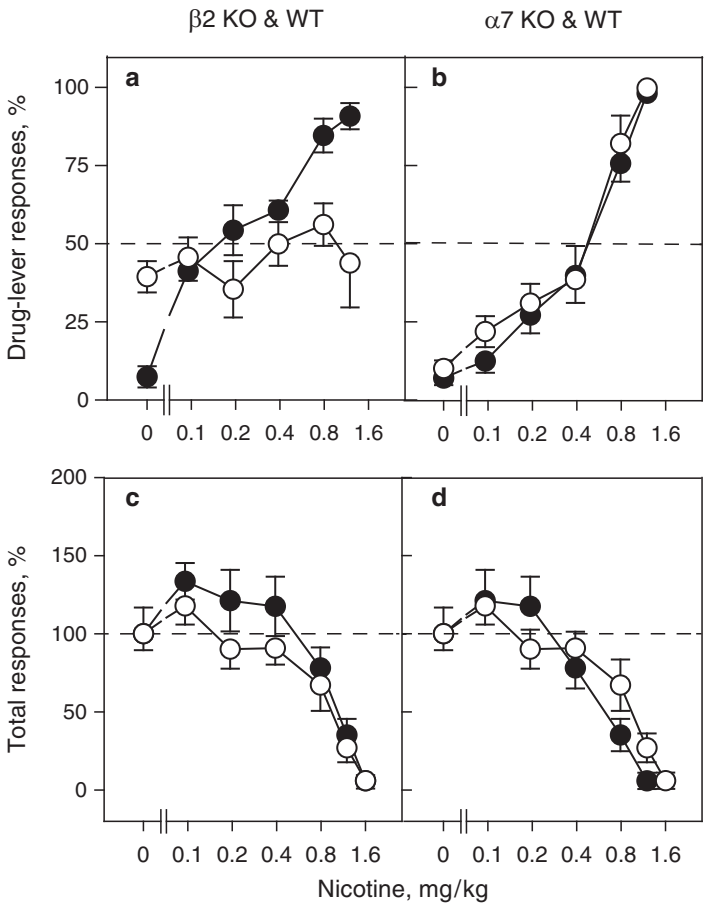

Fig. 5 Dose–response curves for discriminative stimulus effects of nicotine in wild-type (*filled circle*) and knockout (*open circle*) mice trained with nicotine (0.8 mg kg^{-1}). (**a**) shows results for β2 null mutant and control mice from Shoaib et al. (2002) whereas (**b**) shows corresponding results for α7 null mutants and their wild-type controls from Stolerman et al. 2004. Discriminative responding on the drug-appropriate lever is expressed as a percentage of the total numbers of responses on both levers. All data shown as means ± s.e.m. from 5-min extinction tests ($n = 8$–12). Figure 4a was published in Shoaib et al. (2002) The role of nicotinic receptor beta-2 subunits in nicotine discrimination and conditioned taste aversion. Copyright Elsevier; Fig. 4b was published in Stolerman et al. (2004). The role of nicotinic receptor alpha7 subunits in nicotine discrimination. Copyright Elsevier

6 Secondary Pharmacological Mediation

6.1 Dopamine

Investigations on the role of dopamine in nicotine discrimination were triggered by reports that nicotine was able to induce dopamine release both in vitro

(Westfall 1974) and in vivo (Giorguieff-Chesselet et al. 1979; Imperato et al. 1986). Initial studies therefore compared the discriminative effects of nicotine with those of other dopamine-releasing compounds. Rats trained to discriminate nicotine typically showed partial generalisation to amphetamine or other non-selective indirect dopamine agonists such as cocaine (Chance et al. 1977; Desai et al. 1999, 2003; Mansbach et al. 1998; Reavill and Stolerman 1987). Complete generalisation to amphetamine or cocaine has occurred in some experiments (Gasior et al. 1999; Stolerman 1989; Stolerman and Garcha 1989). Both partial and full cross-generalisation with amphetamine and cocaine have also been reported in mice and monkeys (de la Garza and Johanson 1983; Stolerman et al. 2008). Additionally, nicotine potentiated the response to submaximal doses of amphetamine in rats trained to discriminate it (Reavill and Stolerman 1987). Bupropion has also been reported to produce partial or full generalisation with nicotine (Desai et al. 2003; Wiley et al. 2002; Young and Glennon 2002), an effect not seen in other studies (Shoaib et al. 2003). Although the preceding observations were often discussed in terms of dopaminergic mechanisms, it was recognised that neither amphetamine nor bupropion acted exclusively through dopamine. The non-selective MAO inhibitor phenelzine potentiated nicotine discrimination, an effect that may also be a consequence of elevated levels of monoamine neurotransmitters; species differences in the substrate affinities and relative levels of the MAO isozymes limit the direct applicability of these results to humans (Wooters and Bardo 2007).

Studies with directly acting dopamine agonists provided further support for the proposed role of dopamine in nicotine discrimination. There was either partial or full generalisation to the D1 agonists SKF38393, SKF81297 and SKF82958 as well as to the non-selective agonist apomorphine. Such effects were not seen with the D2 agonists bromocriptine and NPA, or with the D3-preferring agonists PD 128,907, 7-OH-DPAT and BP897 (Gasior et al. 1999; Le Foll et al. 2005; Mansbach et al. 1998; Reavill and Stolerman 1987). Initial results with the dopamine uptake blocker GBR−12,909 were negative (Corrigall and Coen 1994) but a later and more comprehensive study yielded full generalisation (Gasior et al. 1999).

Dopamine D1 and D2 antagonists such as haloperidol, SCH23390 and spiperone have usually produced only partial blockade of nicotine discrimination (Corrigall and Coen 1994; Mansbach et al. 1998; Reavill and Stolerman 1987). The non-selective antagonist cis-flupenthixol was ineffective in one study but blocked nicotine in another (Brioni et al. 1994; Desai et al. 2003). However, with rare exceptions such as Desai et al. (2003), the preceding dopamine antagonists were effective only at doses that greatly reduced overall rates of responding. Findings of antagonism by clozapine were suggested as evidence for a possible involvement of D4 receptors (Brioni et al. 1994), but this interpretation cannot be sustained in view of the complex actions of clozapine at dopamine receptors and the negative results obtained with U-101,387, a more selective D4 antagonist (Mansbach et al. 1998). D3 antagonists and partial agonists have also failed to attenuate nicotine discrimination (Le Foll et al. 2005). CGS 10746B, an inhibitor of dopamine release attenuated but did not completely block nicotine discrimination (Schechter and Meehan 1992; Gasior et al. 1999).

Other experiments were built around observations of Mansvelder and McGehee (2000) that α7 as well as β2-containing receptors may contribute to nicotine-stimulated dopamine overflow by examining cross-generalisation between amphetamine and nicotine in α7 knockout mice (Stolerman et al. 2008). The mutant mice showed normal ability to discriminate either nicotine or (+)-amphetamine. However, cross-generalisation between some doses of nicotine and amphetamine was weaker in the knockout than in wild-type mice in both nicotine-trained and amphetamine trained animals. Although tests with a drug more selective than amphetamine are needed, the findings support the concept of a minor dopaminergic element in nicotine discrimination that is mediated via α7 receptors. The contribution of dopamine to the reinforcing effects of nicotine is much clearer and less controversial (Di Chiara, 2000; Picciotto et al. 1998).

A partial reconciliation of some of the above findings can be achieved by considering studies with rats trained to discriminate mixtures of drugs. Such subjects generalised partially or fully to the separate drugs in the mixtures (Stolerman et al. 1987; Mariathasan et al. 1991) and this may explain the relatively positive findings of studies using agonists to test for a dopaminergic element in the nicotine discriminative stimulus, as contrasted with the often negative findings with dopamine antagonists. The studies on drug mixtures indicated that clear evidence for antagonism was not detectable with drugs that blocked only one element in a compound drug stimulus (White and Stolerman 1994; Mariathasan et al. 1997). Insofar as dopamine mediates only one element in a compound, nicotine-produced stimulus, this behavioural mechanism may account for the negative findings in some experiments with dopamine antagonists. As noted by Di Chiara (2000), the effects of dopamine antagonists might be further elucidated by studying their effects on cues elicited by nicotine infused into different brain areas. The absence of reports on the effects of selective neurotoxin-induced lesions of dopaminergic neurones constitutes another major gap in the evidence base.

6.2 Serotonin

There is some overlap between the distribution of nicotine and 5-HT receptors and many studies have indicated that nicotine modulates the functioning of serotonergic systems. Both acute increases in serotonin overflow and chronic downregulation of the synaptic synthesis of 5-HT have been reported (Benwell and Bakfiyr 1979; Ribeiro et al. 1993; Summers and Giacobini 1995). Studies have therefore looked for a possible mediating or modulatory role of serotonergic systems in relation to nicotine discrimination. Earlier studies were mostly driven by the idea that nicotine-facilitated release of 5-HT might play a mediating role in transduction of the action of nicotine at its receptor sites into the perceived discriminative stimulus. However, Schechter and Rosecrans (1972) found that general depletion of central 5-HT has little impact on nicotine discrimination in rats and the antagonist methergoline, that acts non-selectively at 5-HT_1 and 5-HT_2 receptors, failed to attenuate nicotine discrimination (Stolerman et al. 1983). The selective 5-HT_{2A} and 5-HT_{2C}

antagonists M100,907 and SB 242,084, buspirone (a 5-HT_{1A} partial agonist), and 5-HT_3 antagonists (ICS-205930, MDL 72,222 and ondansetron) also failed to attenuate nicotine discrimination (Schechter and Meehan 1992; Stolerman and Garcha 1994; Zaniewska et al. 2007). Interestingly, the 5-HT_6 antagonist MS-245 potentiated the discriminative effect of a sub-threshold dose of nicotine although it did not generalise with or block the effects of nicotine (Young et al. 2006).

Recently, the idea that serotonergic stimulation rather than blockade might weaken nicotine discrimination has been tested. Results with 5-HT_{1A} agonists and partial agonists were negative (Stolerman and Garcha 1994; Batman et al. 2005) but the 5-HT_2 agonists DOI, DOB and MK212 partially or fully blocked nicotine discrimination (Batman et al. 2005). These substances acted at both 5-HT_{2A} and 5-HT_{2C} receptors. Later studies used Ro-60–0175, a relatively selective agonist at 5-HT_{2C} receptors (Millan et al. 1997; Di Matteo et al. 2004); Fig. 6 shows that Ro-60–0175 was not generalised with nicotine but produced a small rightward shift of the dose–response curve for nicotine (Quarta et al. 2007; Zaniewska et al. 2007). Ro-60–0175 was known to inhibit nicotine-stimulated dopamine release in the striatum and nucleus accumbens (Di Matteo et al. 2004; Pierucci et al. 2004), and it was suggested that its effect on nicotine discrimination was mediated through a dopaminergic mechanism. Ro-60–0175 also weakens nicotine self-administration in rats (Grottick et al. 2001). The extensive studies by Zaniewska et al. (2007) showed that the 5-HT_{2A} and 5-HT_{2C} antagonists M100,907 and SB 242,084 reversed the inhibition of nicotine discrimination produced by DOI and Ro-60–0175 respectively. The possibility that DOI and Ro-60–0175 might attenuate nicotine discrimination by a psychological mechanism such as stimulus masking needs to be investigated because both of these substances can themselves produce robust discriminative stimuli (Dekeyne et al. 1999; Schreiber et al. 1994). Nevertheless the studies suggest that activation of either $5HT_{2A}$ or $5HT_{2C}$ receptors may have an important modulatory role in attenuating the nicotine stimulus.

6.3 Glutamate

Relatively few drug discrimination studies have examined the interactions of ligands for glutamate receptors with nicotine. Kim and Brioni (1995) reported that the NMDA antagonist MK-801 (dizocilpine) neither generalised with nor blocked the effects of the training dose of nicotine in rats, a finding confirmed in a later more extensive study that extended the observations by showing the absence of effects of MK-801 on submaximal doses of nicotine (Zakharova et al. 2005). Memantine, another NMDA antagonist, weakly attenuated responses to several doses of nicotine but there was evidence that this less selective compound may have acted directly on nicotinic $\alpha4\beta2$ receptors. An antagonist at metabotropic glutamate mGlu5 receptors (MPEP) also did not generalise with or block the response to the training dose of nicotine. However MPEP did attenuate the response to smaller doses of nicotine and produced a small rightward shift of the nicotine dose–response curve (Zakharova et al. 2005).

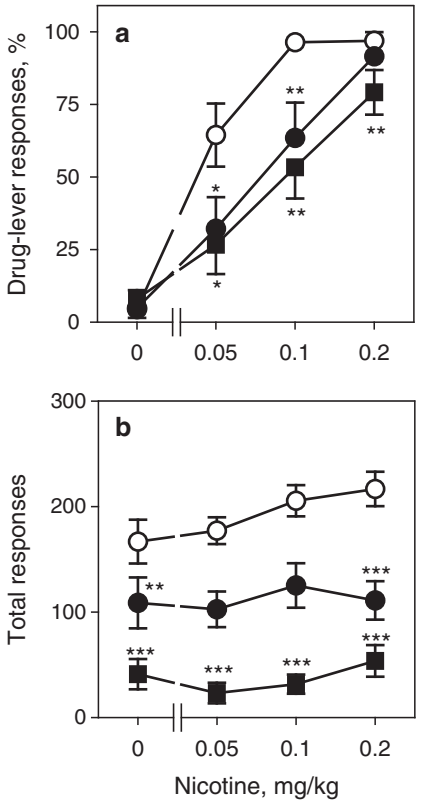

Fig. 6 Influence of Ro-60–0175 at doses of 0.45 mg kg^{-1} (*filled circle*) or 0.9 mg kg^{-1} (*filled square*) on (**a**) the nicotine discriminative stimulus shown as percent responding on the drug-appropriate lever and (**b**) total numbers of responses. Rats ($n = 12$) were trained to discriminate nicotine (0.2 mg kg^{-1}) from saline. Data are shown as means (± s.e.m.) for responding in 5 min extinction tests. Significant effects of Ro-60–0175 as compared with saline at each dose of nicotine are marked ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$, by Dunnett's t-tests). Data for discriminative effects in the tests with 0.9 mg kg^{-1} of Ro-60–0175 are for 7–10 rats due to response suppression in other animals. Reproduced with permission from Fig. 5 in Quarta et al. (2007). The serotonin 2C receptor agonist Ro-60–0175 attenuates effects of nicotine in the five-choice serial reaction time task and in drug discrimination. Copyright Springer

6.4 Cannabinoids

Functional interactions between the nicotinic and cannabinoid systems have been proposed (Cohen et al. 2002) and several studies have tested the applicability of these ideas to nicotine discrimination. However cannabinoid agonists acting at cannabinoid CB_1 and CB_2 receptors have failed to generalise with nicotine (Zaniewska et al. 2006). Results with the anandamide uptake and fatty acid amide hydrolase inhibitors AM-404 and URB 597, that elevate brain concentrations of endogenous cannabinoids, were also negative. Furthermore, neither the CB_1 receptor

antagonist rimonabant nor the CB_2 antagonist SR 144528 attenuated the discriminative stimulus effect of nicotine (Cohen et al. 2002; Le Foll and Goldberg 2004; Zaniewska et al. 2006). Solinas et al. (2006) investigated the effects of nicotinic agents on the discrimination of delta9-tetrahydrocannabinol (THC) in rats. Nicotine potentiated the discriminative effects of small doses of THC (0.3–1 mg kg^{-1}) although it was not generalised with THC. Rimonabant reversed this potentiation of THC discrimination, suggesting that it was at least partly mediated by a release of endogenous cannabinoids. In addition, after administration of URB-59, nicotine produced THC-like discriminative effects that were antagonised by rimonabant. The preceding results suggested that activation of nicotinic receptors modulates the discriminative effects of THC and raise interesting questions about the generality of the effects and whether nicotine can facilitate the reinforcing effects of cannabinoids.

6.5 Opioids

Diverse studies in animal and human subjects suggested that opioids may play a role in some effects of nicotine (e.g. Corrigall et al. 1988; Brauer et al. 1999). A small number of studies have examined the role of opioidergic mechanisms in the nicotine discriminative stimulus. The opioid antagonists naloxone and naltrexone that act predominantly at mu-opioid receptors did not attenuate discrimination of nicotine in a T-maze paradigm (Romano et al. 1981; Overton 1983). The preceding studies examined the effects of the antagonists on the response to the training dose of nicotine only and tests for more subtle attenuation at sub-maximal doses of nicotine were not reported. The effect of the kappa-opioid agonist U50,488 was determined because such agents can attenuate nicotine-stimulated dopamine release, but U50,488 in a range of doses up to 1.25 mg kg^{-1} did not shift the nicotine dose–response curve (Hahn et al. 1999). Therefore, a role for opioid mechanisms in nicotine discrimination remains to be demonstrated.

6.6 Interactions with Caffeine

The innovative studies of Gasior and colleagues showed that chronic exposure to caffeine had striking and complex effects on nicotine discrimination. Caffeine was administered in rats' drinking water at several concentrations. Acquisition of nicotine discrimination was enhanced by consumption of a 0.25 mg ml^{-1} solution of caffeine but not by solutions containing 1.0 or 3.0 mg ml^{-1} of caffeine (Gasior et al. 1999, 2000). The 0.25 mg ml^{-1} solution also markedly increased generalisation to amphetamine and cocaine whereas the 1.0 ml kg^{-1} solution had little effect (Gasior et al. 2000). Contrastingly, the 3.0 mg kg^{-1} concentration of caffeine used by Gasior et al. (1999) blocked generalisation to both amphetamine and cocaine and also to the dopamine reuptake inhibitor GBR-12909. Figure 7 shows the results of Gasior et al. (1999) for cocaine and GBR-12909 at two training doses of nicotine

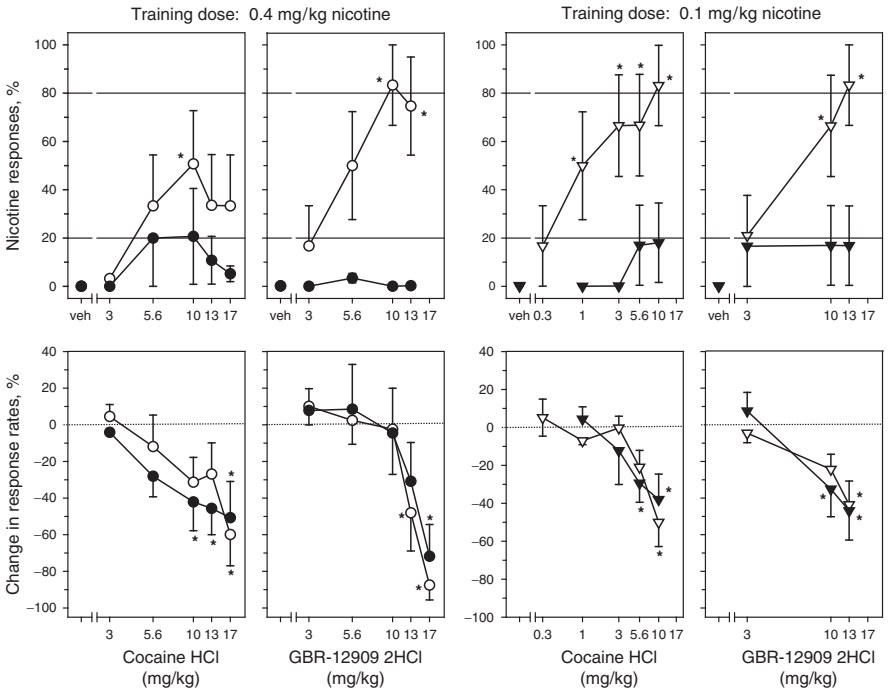

Fig. 7 Dose–response functions for stimulus generalisation of cocaine and GBR-12909 in water- and caffeine-drinking rats ($n = 5$–6). Circles represent performance of rats drinking tap water (*open circle*) or caffeine solutions (*filled circle*) and trained to discriminate nicotine (0.4 mg kg^{-1}) from saline. *Triangles* represent performance of rats drinking tap water (*inverted open triangle*) or caffeine solutions (*filled inverted triangle*) and trained to discriminate nicotine (0.1 mg kg^{-1}) from saline. *Upper sections*, percentage of nicotine-appropriate responding after administration of cocaine, GBR-12909, or vehicle (means ± s.e.m.). *Lower sections*, percentage of change from baseline rates of responding (means ± s.e.m.). *The dashed line* at 0% denotes no change from the individual baseline rate of responding. *Asterisks* represent performance significantly ($p < 0.05$) different from vehicle (Dunnett's test after one-way repeated measures ANOVA). Reproduced with permission from Fig. 5 in Gasior et al. (1999). Acquisition of nicotine discrimination and discriminative stimulus effects of nicotine in rats chronically exposed to caffeine. Copyright The American Society for Pharmacology and Experimental Therapeutics

and thus "depending on the concentration of caffeine, the ability of amphetamine and cocaine to generalise to the nicotine cue changed in a biphasic manner from potentiation to no effect to attenuation in rats chronically exposed to 0.25, 1.0 and 3.0 mg ml^{-1} caffeine concentrations, respectively" (Gasior et al. 2000).

The plasma concentrations of caffeine in the rats exposed to the 0.25 and 1.0 mg ml^{-1} solutions were within the range of those found in typical users of caffeinated beverages. Caffeine may weakly stimulate dopamine mechanisms via its antagonist action at adenosine receptors (Herrera-Marschitz et al. 1988; Casas et al. 1989; Ferre et al. 1992; Fredholm et al. 1999), and in small doses it may have enhanced the dopaminergic component in the nicotine discriminative stimulus through an effect

on the performance of a previously acquired discrimination. This interpretation is supported by evidence that even acute administration of caffeine is sufficient to enhance the discriminative response to a small, sub-threshold dose of nicotine (Gasior et al. 2002). Additionally, during exposure to the 3.0 mg ml^{-1} solution of caffeine, the occurrence of caffeine-stimulated dopaminergic activation in both saline and nicotine training sessions may have masked the value of nicotine-induced dopaminergic activation as a cue for the acquisition of discriminative responding, thus explaining the absence of generalisation to psychomotor stimulants and GBR-12909 under such conditions. Interpretations could also be based on the ability of large doses of caffeine to inhibit phosphodiesterase activity (Fredholm et al. 1999). Studies are needed to distinguish between the different explanations for the dose-related effects of caffeine on nicotine discrimination. Further investigations in this area are especially desirable in view of associations between the use of the two substances in human populations.

7 Conclusions

Drug discrimination studies have contributed importantly to characterising the nicotinic receptor subtypes that mediate its behavioural effects. Insofar as discriminative stimulus effects model or are analogous to subjective effects, the results may be relevant to the effects of the drug sought by tobacco users. In this respect, many conclusions from the discrimination studies are strikingly similar to those from investigations of the positive reinforcing properties of nicotine in self-administration studies and, where data are available, of its actions in related procedures assessing conditioned place preferences and thresholds for intra-cranial electrical stimulation of brain reward systems (see chapter on nicotine self-administration). Each of these techniques has its own strengths and weaknesses, and conclusions are most convincing when supported by data from all of them. Discrimination studies have used both classical pharmacological approaches based on ligands with differing selectivity for nicotinic receptor subtypes and animals with targeted deletions of nicotinic receptor subtypes. Studies with these different approaches concur in their support for the view that the heteromeric $\alpha4\beta2$ receptor is the predominant receptor subtype at which the major psychoactive effects of nicotine originate (Sect. 5 above). The available data do not distinguish the possible roles of some other minor heteromeric subtypes although there is broad agreement that actions at homomeric $\alpha7$ receptors contribute little to the nicotine stimulus complex.

The studies reviewed in Sect. 6.1 above also provide numerous findings suggesting that dopamine released downstream from the nicotinic receptors acts as a mediator of nicotine discrimination. Nevertheless, the findings from studies with dopamine antagonists provide evidence that dopamine-related effects comprise only one element in the nicotine-induced stimulus complex. This conclusion appears to contrast with studies on the positive reinforcing property of nicotine where dopamine appears to play a crucial mediating role, as shown not only by studies with

dopamine antagonists (Corrigall and Coen 1991; David et al. 2006), but also by the elegant demonstration of the abolition of self-administration after 6-OHDA lesions of the mesolimbic dopamine system in rats (Corrigall et al. 1992). In this respect, the nature of the discriminative and reinforcing effects of nicotine, and the mechanisms through which they are mediated, appear to be similar rather than identical. Di Chiara (2000) suggested a more profound distinction by presenting a carefully argued case that the nicotine discriminative stimulus is more closely related to its aversive than its positive reinforcing effects. Nicotine has indeed been shown to serve as a potent stimulus in punishment, negative reinforcement and taste conditioning paradigms for assessing aversive drug actions. (Goldberg and Spealman 1983; Kumar et al. 1983; Spealman 1983). Some findings do not appear to support Di Chiara's hypothesis; notably, chronic (persistent) tolerance develops to some aversive effects of nicotine (Stolerman 1999) but it has not been shown clearly for the nicotine discriminative stimulus (Shoaib et al. 1997). Nevertheless, the provocative suggestion of Di Chiara (2000) deserves further investigation through studies comparing the neurobiological mechanisms for the discriminative and reinforcing stimulus effects of nicotine.

In addition to a role for dopamine, evidence has accumulated for interactions with the 5-HT, glutamate and cannabinoid systems in the expression of the nicotine discriminative stimulus (Sects. 6.2–6.4). These areas have been researched less intensively, but some positive findings support the case for further work. Notably, the studies strongly suggested that pharmacological activation of some 5-HT_2 receptor subtypes attenuates expression of the nicotine stimulus. Interestingly, findings suggest that activation of nicotinic receptors modulates the discriminative effects of THC but the reverse was not the case; these observations raise interesting questions about the generality of the effects and whether nicotine can facilitate the reinforcing effects of cannabinoids. If this is the case, then there would be a strong implication that human cigarette smoking constitutes a predisposing factor or "gateway" (Jessup 1996) for increased cannabinoid intake among those individuals that initiate the consumption of marijuana. The limited amount of evidence currently available suggests that glutamatergic neurotransmission may have no more than a minor role in nicotine discrimination, but the evidence from studies with the mGluR5 antagonist MPEP supports findings that metabotropic glutamate receptors are involved in the positive reinforcing effect of nicotine (Paterson et al. 2003). More extensive studies with a wider range of glutamate receptor ligands are needed before firm conclusions can be reached. In contrast, there does not seem to be evidence that opioid mechanisms have a role in nicotine discrimination (Sect. 6.5) or self-administration (DeNoble and Mele 2006).

Many studies reviewed by Tanda and Goldberg (2000) have found evidence for interactions of nicotine with caffeine and drug discrimination studies have further expanded these investigations (Sect. 6.6). The bidirectional, dose-dependent nature of caffeine effects on nicotine discrimination invites comparisons with parallel studies of nicotine self-administration. However, whereas very large chronic doses of caffeine that were much above those obtained by consumers of caffeinated beverages potentiated nicotine self-administration (Shoaib et al. 1999), there is a

surprising lack of similar animal studies using more relevant doses of caffeine. Research in human subjects has to date failed to find evidence that caffeine influences the discriminative or reinforcing stimulus effects of nicotine (Perkins et al. 2005).

The investigations reviewed above illustrate the exceptionally valuable role of the discrimination procedure for defining the neuropharmacological mode of action of nicotine. Such work has benefited substantially from the excellent understanding of some key procedural factors that influence the development and expression of nicotine discrimination (Sect. 3). Notably, the effects of establishing nicotine discriminations using different training doses, times of administration and schedules of reinforcement are reasonably well understood, as is the way in which responses to different elements in a drug-induced state interact to produce a complex discriminative stimulus.

It has often been assumed that there is a close relationship between the reinforcing and discriminative stimulus effects of drugs generally, including nicotine. The functional roles of drugs in the two types of procedure are by definition different, but it remains possible that the perceived effects of the substance are similar if not identical. In each case such effects may be seen as having several, possibly inter-related elements that together comprise the drug-induced stimulus complex. However, analysis of these stimulus complexes in terms of subjective drug experiences is problematic and difficult even for human subjects and even more formidable for animals. Any underlying similarity in the reinforcing and discriminative effects of drugs can therefore probably be determined by comparing the neuropharmacological mechanisms and brain sites implicated in the two phenomena. The evidence discussed above suggests rather strongly that both stimulus effects originate predominantly at similar subtypes of heteromeric nicotinic receptors (Sect. 5.2 above). Administering either a glutamate mGlu5 antagonist or a 5-HT_{2C} agonist attenuates both stimulus properties of nicotine (Sects. 6.2 and 6.3). However, while intact dopaminergic neurotransmission in the mesolimbic system seems to critical for expression of nicotine's reinforcing effect, it appears to have a relatively minor role in nicotine discrimination (Sect. 6.1). The neuroanatomical origins of the effects may also differ; reinforcing effects have been shown to originate largely in the ventral tegmental area of the midbrain whereas the strongest evidence for discriminative effects supports actions in the prefrontal cortex (Sect. 4.2). However, the evidence base for the preceding comparisons has many limitations and studies designed to fill some of the major gaps will be valuable, such as studies directly comparing the specific lesions of different transmitter systems and brain regions. On the basis of presently available evidence it can be speculated that the neuropharmacological basis for the discriminative and reinforcing stimulus effects of nicotine are similar rather than identical and that neither paradigm can serve as a reliable surrogate for the other.

In summary, investigations of the nicotine discriminative stimulus complex have contributed to our present understanding of its psychopharmacological properties by defining their origin at specific subtypes of nicotinic receptor and the role of diverse neurotransmitter systems as mediating and modulating mechanisms. The evidence strongly supports central sites as the origins of the nicotine stimulus, and these are likely to be located in the mesocorticolimbic dopaminergic neurons; the

medial prefrontal cortex is primarily involved, with the nAcc and VTA of secondary importance, while another element of the complex stimulus may arise in the dorsal hippocampus (Sect. 4 above). Additionally, it appears that interactions of nicotine with the dopamine, serotonin, cannabinoid and probably glutamate systems all contribute to the final perceived stimulus. A very limited number of investigations have failed to find a role for opioid systems. It is particularly interesting that acute and chronic exposure to caffeine produce quantitative and qualitative changes in the characteristics of the nicotine stimulus. Additional information about interactions of nicotine with caffeine and cannabinoids may strengthen hypotheses whereby the use of one substance serves as a gateway in sequential shifts of the target substance for drug-seeking behaviour, with profound implications for the human use of the substances concerned.

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Effects of Nicotine in Experimental Animals and Humans: An Update on Addictive Properties

Bernard Le Foll and Steven R. Goldberg

Contents

Abstract Tobacco use through cigarette smoking is the leading preventable cause of death in the developed world. Nicotine, a psychoactive component of tobacco, appears to play a major role in tobacco dependence, but the reinforcing effects of nicotine have often been difficult to demonstrate directly in controlled studies with laboratory animals or human subjects. Here we update our earlier review published in Psychopharmacology (Berl) in 2006 on findings obtained with various procedures developed to study dependence-related behavioral effects of nicotine in experimental animals and humans. Results obtained with drug self-administration, conditioned place preference, subjective reports of nicotine effects and nicotine discrimination indicate that nicotine can function as an effective reinforcer of drug-seeking and drug-taking behavior both in experimental animals and humans under appropriate

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conditions. Interruption of chronic nicotine exposure produces ratings of drug withdrawal and withdrawal symptoms that may contribute to relapse. Difficulties encountered in demonstrating reinforcing effects of nicotine under some conditions, relative to other drugs of abuse, may be due to weaker primary reinforcing effects of nicotine, to aversive effects produced by nicotine, or to a more critical contribution of environmental stimuli to the maintenance of drug-seeking and drug-taking behavior with nicotine than with other drugs of abuse. Several recent reports suggest that other chemical substances inhaled along with nicotine in tobacco smoke may play a role in sustaining smoking behavior. However, conflicting results have been obtained with mice and rats and these findings have not yet been validated in nonhuman primates or human subjects. Taken together, these findings suggest that nicotine acts as a typical drug of abuse in experimental animals and humans in appropriate situations.

1 Introduction

Tobacco smoking is presently estimated to cause 20% of all deaths in developed countries. As with other types of drug dependence, tobacco dependence is described as a chronic, relapsing disorder in which compulsive drug-seeking and drug-taking behavior persist despite negative consequences and the motivation to quit. The highly addictive effects of tobacco are exemplified by the great difficulty in quitting smoking. Although most smokers want to stop, only a small percent succeed. It is now becoming clear that continued tobacco use induces adaptive changes in the central nervous system that lead to drug dependence (American Psychiatric Association 2000). Nicotine, the major psychoactive component of tobacco, is thought to play a critical role in tobacco dependence through its actions as a reinforcer of drug-seeking and drug-taking behavior (Fiore et al. 2000; Henningfield and Goldberg 1983a, b; Stolerman and Shoaib 1991). Nevertheless, tobacco smoke contains several hundred other chemical substances, some of which have psychoactive effects or may enhance the psychoactive effects of nicotine, and these other substances may contribute to the reinforcing effects of tobacco smoking (Fowler et al. 1996a, b). Indeed, the reinforcing effects of nicotine have often been difficult to demonstrate directly in past controlled studies with either laboratory animals or humans as experimental subjects. As a result, there has been some controversy in the literature about the validity of previous findings that nicotine can produce reinforcing effects in experimental animals or human subjects (Dar and Frenk 2002, 2004; Robinson and Pritchard 1992).

A variety of laboratory animal models are available to study the cardinal features of drug dependence (Deroche-Gamonet et al. 2004; Everitt and Robbins 2000; Goldberg et al. 1975; Goldberg et al. 1981a, b, 1975, 1979; Katz and Goldberg 1988; Le Foll and Goldberg 2005a–d; Markou et al. 1993; Schindler et al. 2002; Schuster and Woods 1968; Spealman and Goldberg 1978; Vanderschuren and Everitt 2004). The effects of nicotine have been evaluated using animal models for studying the

reinforcing effects of drug injections (intravenous drug self-administration and conditioned place preference (CPP) procedures), the subjective responses to administered drugs (drug discrimination), the withdrawal states (including behavioral disturbances) that are associated with abrupt termination of chronic drug exposure (smoking cessation or administration of selective antagonists after chronic exposure), and relapse phenomena (reinstatement of extinguished drug-seeking behavior induced by stress, drug-associated cues, or drug priming). Most of these experimental studies have used rodents (rats and mice) as subjects, but results are available from studies using other animal species (monkeys and dogs) and human volunteers as subjects. We will first summarize the main experimental procedures used to assess these effects of nicotine and then review the preclinical and clinical findings obtained with nicotine using these procedures. Since previous review articles have already provide detailed comparisons of the effects of nicotine in animals and humans (Henningfield and Golberg 1983a, b; Le Foll and Goldberg 2006; Rose and Corrigall 1997; Stolerman 1999), we focus here on the most recent important findings obtained with nicotine in animals and humans.

2 Experimental Procedures for Studying Nicotine's Effects

2.1 Intravenous drug self-administration

Natural rewards, such as water or food and drugs of abuse may serve as positive reinforcers under appropriate conditions. For example, to assess the reinforcing effects of food, a food-deprived animal can be placed in a sound-attenuating chamber containing stimulus lights, response levers and a device for dispensing food pellets. Lever-pressing responses will occur with increasing frequency when they result in delivery of food pellets, which, therefore, serve as positive reinforcers under these conditions. With intravenous drug self-administration procedures, a catheter implanted in a jugular vein allows the animal to intravenously self-administer a small amount of drug by pressing a lever. The administration of drug constitutes the event that positively reinforces the lever-pressing behavior and reward is inferred if the frequency of responding subsequently increases (thus, defining reinforcement). With these behavioral procedures, stimuli such as a light or tone are often associated with delivery of the reinforcer. It has been argued that in many instances these stimuli are not neutral, but themselves have the potential to produce weak reinforcing effects and there is accumulating evidence that nicotine exposure can increase their motivational value (i.e., they may become more effective reinforcers) (Chaudhri et al. 2006a). These stimuli, or 'cues', can also progressively gain motivational value by Pavlovian conditioning and associative learning processes. In either case, environmental stimuli can acquire the ability to facilitate the maintenance of drug-seeking and drug-taking behavior and also reinstate drug-seeking behavior that has been extinguished (Arroyo et al. 1999; de Wit and Stewart 1981; Goldberg 1975; Goldberg et al. 1975, 1981a, b, 1983; Le Foll and Goldberg 2005b;

Meil and See 1996; Self and Nestler 1988; Stewart 1983), and may become critical determinants of reinforcement of drug-taking behavior by nicotine administration.

Various schedules of reinforcement have been employed to study drug self-administration behavior. Two of the most commonly used are fixed-ratio and progressive-ratio schedules of intravenous drug injection. Under a fixed-ratio schedule of intravenous drug injection, the subject must make a fixed number of responses (lever press or pull or nose-poke) in order to obtain each injection of drug (e.g., one lever press for a fixed-ratio one, i.e., FR1, schedule). In contrast, under a progressive-ratio schedule of intravenous drug injection, the number of responses the subject must make to obtain successive drug injections (the ratio value) increases progressively until the subject fails to make the required number of responses (Hodos 1961). The highest ratio reached before responding ceases (the 'breaking point') is thought to reflect the reinforcing effectiveness of the drug (Donny et al. 1999; Le Foll et al. 2007b). Intravenous self-administration studies have repeatedly shown that most drugs considered to be addictive in humans can serve as positive reinforcers for laboratory rats and monkeys, whereas non-addictive drugs have given negative results in the great majority of cases (Balster 1992; Katz and Goldberg 1988). Once an animal has learned to intravenously self-administer a drug, the influences of drug priming, stressors or presentation of drug-associated stimuli on drug self-administration behavior or relapse to extinguished drug-seeking behavior provide useful measures for studying the behavioral aspects of drug dependence (see Shalev et al. 2002 for a review). Interestingly, nicotine self-administration has also been studied under second-order schedules of reinforcement in nonhuman primates. See Everitt and Robbins (2000) and Schindler et al. (2002) for reviews on those schedules. In this paradigm, animals first learn to self-administer the drug intravenously. Each drug infusion is made contingent upon a response on a lever and is paired with a light stimulus, which becomes the conditioned stimulus (C.S.). During acquisition of the behavior, the number of lever responses required to produce the C.S. is progressively increased, as well as the number of C.S. presentations that have to be produced before the C.S. is paired with a drug infusion. The C.S. progressively gains motivational salience and, as a conditioned reinforcer, maintains and controls drug-seeking behavior (Goldberg and Gardner 1981; Goldberg et al. 1981a, b).

2.2 Drug-induced conditioned place preferences

Another experimental animal model for exploring the reinforcing effects of drugs of abuse is the conditioned place preference (CPP) procedure. A distinctive environment (e.g., one compartment of a two- or three-compartment apparatus) is paired repeatedly with administration of a drug, and a different environment is repeatedly associated with administration of vehicle. CPP occurs when repeated administration of a drug in this particular environment results in the ability of that environment to elicit approach behavior and increased time contact (place preference) in the

absence of the previously administered drug. It has been argued that CPP, like drug self-administration and a number of related phenomena, is an example of dopamine-mediated incentive learning and that the approach behavior and increased time spent by animals in a drug-paired environment can be considered a measure of drug-seeking behavior and the reinforcing effects of drugs (Bardo and Bevins 2000; Le Foll and Goldberg 2005d). CPP has been demonstrated for most drugs of abuse, as well as for natural reinforcers such as food. The acquisition of a drug-induced CPP is likely to be correlated with other reinforcing effects of abused drugs, whereas its expression reflects the influence on the behavior of environmental stimuli previously associated with a drug's effects.

2.3 Drug discrimination

Humans exposed to psychoactive drugs report characteristic subjective effects; drug-discrimination procedures in rats and monkeys are extensively used as animal models of these subjective reports of drug effects in humans. The ability to perceive and identify the characteristic interoceptive effects of abused drugs is thought to play a critical role in drug-seeking, encouraging the development of this behavior and directing it towards one substance rather than another, on the basis of relative potencies and subjective effects (Colpaert 1999; Stolerman and Shoaib 1991). These interoceptive subjective effects of drugs are most frequently assessed in humans through the use of subject-rating scales, and correlated changes in behavior are frequently assessed using performance–assessment tasks. In animals, the interoceptive effects of drugs can serve as discriminative stimuli indicating how to obtain a reinforcer such as a food pellet or how to avoid an electric shock (Solinas et al. 2006). For example, animals can be trained under a discrete-trial schedule of food-pellet delivery or stimulus-shock termination to respond on one lever after an injection of a training dose of nicotine and on the other lever after an injection of vehicle. Once animals learn to reliably make this discrimination, the discriminative effects of different drugs or different nicotine doses can be compared and the modulation of subjective effects of nicotine by various pharmacological treatments can be measured (Le Foll and Goldberg 2004; Le Foll et al. 2005c). This procedure works well with nicotine in rats (Rosecrans 1979; Stolerman 1989) (Fig. 2a), mice (Shoaib et al. 2002; Stolerman et al. 1999), and squirrel monkeys (Takada et al. 1988) and has also been used in human subjects by using nasal sprays containing either nicotine or placebo (Perkins et al. 1996).

2.4 Measurement of withdrawal disturbances

Abrupt cessation of exposure to most drugs of abuse leads to withdrawal signs and symptoms in humans (American Psychiatric Association 2000) and these can be

measured in humans through reports by subjects using standardized rating scales and through reports of trained observers (Hughes et al. 1991). Animal models have been developed to evaluate the physical signs, as well as the behavioral consequences, of inferred emotional disturbances following cessation of exposure to drugs of abuse. In these procedures, the animals are frequently implanted chronically with minipumps that deliver the drug continuously. Cessation is produced either by the removal of the pump or by the injection of a specific antagonist (Malin et al. 1992; Watkins et al. 2000).

3 Effects of Nicotine in Experimental Animals and Humans

3.1 Reinforcing Effects of Nicotine in Experimental Animals

Intravenous self-administration of a psychoactive drug is generally considered to be the most direct measure of a drug's reinforcing effects. Although intravenous drug self-administration procedures generally work well with psychostimulants and opioids over a relatively wide range of conditions, the conditions under which nicotine maintains nicotine self-administration behavior appear to be more limited. There have been criticisms in the past of the experimental conditions that were used by some investigators to study the reinforcing properties of nicotine in experimental animals. Among the confounding factors cited, we can mention here the omission of controls for general activation, insufficient consideration of secondary reinforcement processes, the use of food-deprived animals, or the exclusion of animals. Our recent analysis of previously published studies performed with the intravenous nicotine self-administration paradigm in nonhuman primates also revealed that most of these studies do not support the conclusion that nicotine, by itself and in the absence of setting conditions, can function as an effective reinforcing agent (Le Foll et al. 2007b). Specific conditions, such as automatic nicotine infusions, previous self-administration of other drugs or food, or food-deprivation, were often employed to demonstrate that nicotine could maintain significant self-administration behavior in nonhuman primates (Le Foll et al. 2007b). In addition, these studies with nicotine self-administration in nonhuman primates often used experimental conditions, such as very slow injection speeds or pretraining on other drugs of abuse, which may not have been optimum for demonstrating reinforcing effects of nicotine. A clear demonstration of the reinforcing effects of nicotine in nonhuman primates has recently been reported (Le Foll et al. 2007b). This study was performed with experimentally naive squirrel monkeys that had no history of exposure to other drugs of abuse, no history of drug self-administration, and had not been previously trained to respond for food. Due to the growing literature obtained in rodents suggesting that nicotine may act by increasing the motivational value of environmental stimuli associated with its effects, brief light stimuli were associated with each completion of the FR response requirement on both active and inactive levers. During the

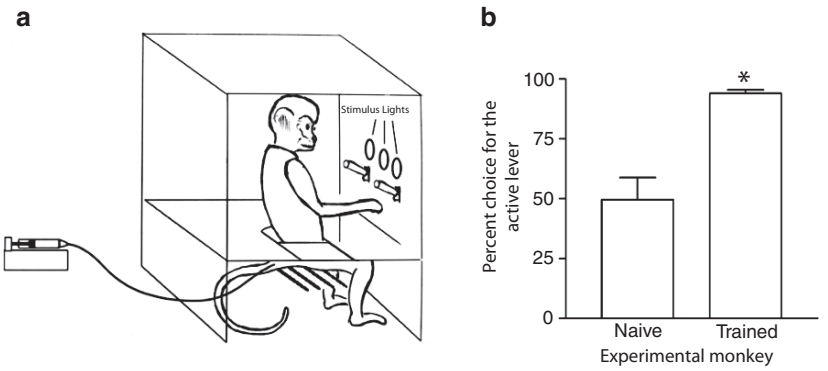

Fig. 1 Active choice of intravenous nicotine in experimentally naive squirrel monkeys. **a** Monkeys sat in chambers equipped with two levers and distinctly colored light stimuli above the levers. Completion of the response requirement (the ratio) on the active lever produced a brief 2-s presentation of a light stimulus and an intravenous injection of nicotine followed by a timeout (TO) period of 5–60 s. Completion of the ratio requirement on the inactive lever resulted in presentation of a brief 2-s light stimulus of a different color but no injection. The fixed-ratio (FR) response requirement was gradually increased over successive sessions from one to ten (FR 1–FR 10). **b** Mean percentage choice for responding on the active lever by monkeys when they were experimentally naive (first week under a FR 1 schedule) and when they had learned to self-administer nicotine under the FR 10, TO 60 s schedule (first week under the FR 10 schedule). $^{*}P < 0.01$, compared to the first week of training. From Le Foll et al. (2007b)

first week of acquisition, no preference was noted for responding on the active versus the inactive lever (percentage choice on the active lever was $49.6 \pm 9.3\%$, as expected by chance) (Fig. 1b). However, over repeated sessions the monkeys developed a strong preference for responding on the active lever compared to the inactive lever ($P < 0.01$) and responding on the inactive lever dropped to negligible levels (Fig. 1b). This shift of responding toward the nicotine-associated lever clearly demonstrates an active choice by the monkeys towards a responding that leads to nicotine delivery.

Once responding was initiated, nicotine clearly maintained self-administration behavior at high levels in squirrel monkeys (Fig. 2), compared to saline vehicle. The reinforcing effects of nicotine appear to be particularly pronounced in squirrel monkeys (Le Foll et al. 2007b) allowing persistent maintenance of nicotine self-administration behavior under fixed-interval (Spealman et al. 1981a, b), second-order (Goldberg et al. 1981a, b), fixed-ratio (Le Foll et al. 2007b; Sannerud et al. 1994), and progressive-ratio (Le Foll et al. 2007b) schedules of intravenous drug injection. In the second-order and progressive-ratio experiments, the monkeys pressed up to 600 times on a lever to obtain a single injection of nicotine (Goldberg et al. 1981a, b; Le Foll et al. 2007b) demonstrating the high motivational value of nicotine that had developed in those experienced animals. In contrast, rates of responding maintained by intravenous nicotine injections in rhesus monkeys and baboons have usually been quite low (Ator and Griffiths 1983; Deneau and Inoki 1967; Goldberg et al. 1981b; Slifer and Balster 1985; Wakasa et al. 1995). These results

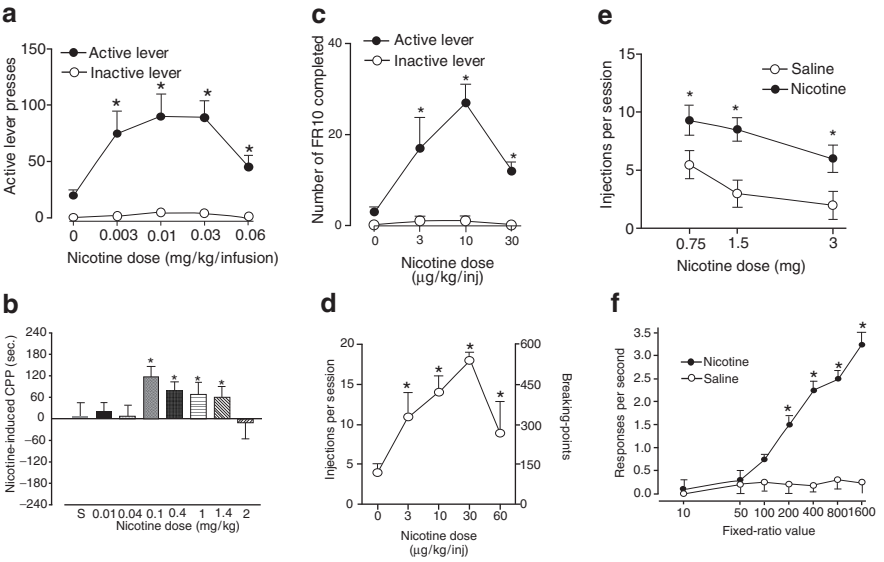

Fig. 2 Reinforcing effects of nicotine in rats (**a,b**), non human-primates (**c,d**) and humans (**e,f**). **a** During repeated sessions, rats learned to press a lever to self-administer intravenous injections of nicotine; light stimuli were paired with each drug infusion. Results are expressed as mean (±SEM) of number of responses on the active and inactive lever. Responding was higher on the active lever compared to the inactive lever. From Corrigall and Coen (1989). **b** Nicotine-induced conditioned place preferences (CPP) over a large range of doses in rats. Over repeated sessions, rats were either injected subcutaneously with nicotine and then placed in one environment, or injected with saline and placed in the other environment. In a nicotine-free state, the animal was then allowed access to both environments during a test session without injection, and the amount of time spent in each environment was recorded. From Le Foll and Goldberg (2005d). **c, d** Nicotine self-administration behavior in squirrel monkeys. Number of ratios completed on the active and inactive levers per session under fixed-ratio (**c**) and progressive-ratio (**d**) schedules are shown as a function of injection dose of nicotine ($n = 5$). From Le Foll et al. (2007b). **e** Human subjects learned to respond on levers to intravenously self-administer nicotine or saline. A light stimulus was paired with each injection. The number of self-administered nicotine injections was consistently higher than the number of self-administered saline injections (**e**) and rate of responding was significantly higher for nicotine than for saline when the number of responses needed to produce an injection was high (**f**). Results are expressed as the mean (±SEM) number of injections per session (**e**) or as a function of the number of responses required for each injection of nicotine or saline (fixed-ratio value) (**f**). From Harvey et al. (2004)

suggest that there may be species differences, although other interpretations are possible since the experimental conditions were not strictly comparable (see Le Foll et al. 2007b for a summary). Similar differences between species have also been reported in rodents. The rate of responding maintained by nicotine is higher in rats (Corrigall and Coen 1989; DeNoble and Mele 2005; Donny et al. 1995) than in mice (Martellotta et al. 1995; Paterson et al. 2003; Rasmussen and Swedberg 1998; Stolerman et al. 1999) (Fig 2a), although this might be related to the greater number of experiments that have been conducted with rats and, thus, the better information

about appropriate experimental conditions that is available. Moreover, findings have not been consistent across or within studies with rats (Brower et al. 2002; Shoaib et al. 1997), where strain differences are likely (Brower et al. 2002; Shoaib et al. 1997). It should be noted that several laboratories are now reporting significant and consistent nicotine self-administration behavior in rats, findings that likely reflect the reliability of the results that can be obtained across laboratories when nicotine is used in specific conditions.

Intravenous nicotine self-administration is usually studied under conditions where availability of injections is restricted by timeout periods ranging from several seconds to several minutes between injections and with daily sessions of short duration (Corrigall and Coen 1989), or under conditions of prolonged access to nicotine (O'Dell et al. 2007; O'Dell et al. 2007; Valentine et al. 1997). In contrast to cocaine, where intake progressively increases after prolonged access to the drug (Ahmed and Koob 1998; Paterson and Markou 2003), no escalation in intake has been found after prolonged access to nicotine (Paterson and Markou 2004), even after periods of time ranging up to 2 years in recent squirrel monkey experiments (Le Foll et al. 2007b). Several studies suggest that rates of responding maintained by nicotine may be less than rates of responding maintained by cocaine when the amount of work required to obtain injections is increased in animals using progressive-ratio schedules (Goldberg and Henningfield 1988; Rasmussen and Swedberg 1998; Risner and Goldberg 1983) or that speed of acquisition of self-administration behavior may be slower than that with other drugs of abuse (Shoaib et al. 1997). However, some investigators have reported similar rates of responding for nicotine and other drugs of abuse in rodents (Paterson et al. 2004; Paterson and Markou 2003) and squirrel monkeys (Le Foll et al. 2007b; Sannerud et al. 1994; Spealman and Goldberg 1982). Nevertheless, existing studies that have directly compared the reinforcing effects of nicotine to those of cocaine using progressive-ratio or choice schedules in the same animals, clearly suggest that the reinforcing effects of nicotine are weaker under progressive-ratio schedules of reinforcement (Manzardo et al. 2002; Risner and Goldberg 1983) and that animals tend to prefer cocaine over nicotine when given the access to both drugs during the same session (Manzardo et al. 2002).

The ability of nicotine to induce CPP has also been frequently studied (Fig. 2). In the CPP procedure, animals are tested in a drug-free state to determine whether they prefer an environment previously associated with the effects of nicotine as compared to an environment previously associated with effects of saline vehicle. Thus, this procedure relies on the capacity of stimuli associated with nicotine's effects to elicit approach responses and increased time spent in the environment associated with nicotine's effects and is used as a measure of reinforcing effects. Nicotine has been shown to induce CPP across a large range of doses in some experiments (Fig. 2b), but the magnitude of the effect is generally small and affected by environmental stimuli or previous handling history (Forget et al. 2005; Grabus et al. 2006; Le Foll and Goldberg 2005b, d), suggesting that the reinforcing effects of nicotine may be weaker that those of other drugs of abuse. Nicotine also produced aversive effects at high dose in some, but not all, studies (Grabus et al. 2006; Le Foll and Goldberg 2005d). It should be noted that nicotine lowers intracranial self-

stimulation reward thresholds, as assessed by the intracranial self-stimulation paradigm, an effect that indicates rewarding effects of nicotine in rodents (Huston-Lyons and Kornetsky 1992).

Experimental variables such as nicotine dose, handling history or environmental cues influence the reinforcing effects of nicotine both in the intravenous self-administration and the CPP procedures (Donny et al. 1998; Grabus et al. 2006; Le Foll and Goldberg 2005b, d). It appears, for example, that adolescent rats, food-deprived animals, and rats previously exposed to nicotine are more likely to acquire intravenous nicotine self-administration behavior or to develop nicotine-induced CPP, compared to rats that are not food-deprived or not previously exposed to nicotine (Adriani et al. 2003; Belluzzi et al. 2004a; Corrigall and Coen 1989; Shoaib et al. 1997, 1994; Vastola et al. 2002). However, the most important variable appears to be environmental stimuli that are repeatedly associated with nicotine injection or marginally reinforcing stimuli whose effects are facilitated by nicotine exposure.

An extensive literature suggests that Pavlovian associative conditioning processes are implicated in the acquisition of motivational value by initially neutral stimuli that are repeatedly paired with the effects of drugs of abuse. In an early paper with monkeys, published in 1981, it was first suggested that environmental stimuli associated with nicotine administration are critical for the maintenance of nicotine-seeking behavior (Goldberg et al. 1981a). During these experiments, a light stimulus was repeatedly paired with nicotine delivery. Although responding ultimately depended on injections of nicotine, the brief light stimulus associated with injections played an important role in the maintenance of persistent responding, since rates of responding were about twice as high when the brief light was presented as when it was absent (Goldberg et al. 1981a).

The critical role played by environmental stimuli in the reinforcing effects of nicotine has recently been demonstrated in rodents. See Caggiula et al. (2002) and Le Foll and Goldberg (2005b) for detailed analyse). In those experiments, discontinuing presentation of environmental stimuli associated with intravenous nicotine injection decreased self-administration behavior almost as effectively as the removal of nicotine itself, indicating their critical role in sustaining drug-taking behavior (Caggiula et al. 2002, 2001; Donny et al. 2003). Moreover, in some experiments with rats (Cohen et al. 2005) and squirrel monkeys (Le Foll et al. 2007b), the responding maintained by nicotine-associated light stimuli was equal to the responding maintained by nicotine itself. In addition, the contingent presentation of environmental light stimuli was able to maintain responding for a prolonged period of time in rats (Cohen et al. 2005) and squirrel monkeys (Le Foll et al. 2007b), demonstrating their persistent nature and their high motivational value. Finally, the use of behavioral procedures that do not have environmental stimuli directly paired with nicotine delivery has been reported to result in very low levels of drug-taking behavior in experiments with drug-naive mice (Paterson et al. 2003) and rats (Donny et al. 2003).

Nicotine, like other psychostimulant drugs (Hill 1970), also produces unconditioned effects that increase the ability of nondrug environmental stimuli to serve as reinforcers, independently of any direct temporal association between nicotine ad-

ministration and stimulus presentation (Caggiula et al. 2002; Chaudhri et al. 2006b; 2007; Olausson et al. 2003, 2004; Palmatier et al. 2007a, b). As an example, in some experiments, noncontingent nicotine, whether delivered as discrete injections based on a pattern of self-administered nicotine or as a continuous infusion, increased response rates maintained by the visual stimulus. There were no significant differences in responding by animals that received contingent as compared with noncontingent nicotine when a visual stimulus was available. Interestingly, operant behavior was equally attenuated and reinstated by the removal and subsequent replacement of contingent and noncontingent nicotine. Although nicotine supported self-administration in the absence of response-contingent, nicotine-paired stimuli, response rates were drastically reduced compared with nicotine self-administration with the visual stimulus (Donny et al. 2003). These experiments suggest that nicotine influences operant behavior in two ways: by acting as a primary reinforcer when it is contingent upon behavior, and by directly potentiating the reinforcing properties of other stimuli through a nonassociative mechanism. It is still unclear whether both processes occur concurrently in smokers, magnifying the role of associated environmental stimuli in nicotine self-administration and tobacco dependence, or whether one process predominates. Interestingly, these conditioning processes may also occur with sensorimotor stimuli of tobacco smoke (Rose 2003b, 2000) and this could explain the reduction in subjective reports of tobacco craving, desire to smoke, and tobacco withdrawal that are produced by placebo cigarettes in smokers (Butschky et al. 1995; Robinson et al. 2000).

3.2 Reinforcing Effects of Nicotine in Humans

Critical variables determining whether or not nicotine functions effectively as a reinforcer of drug-seeking and drug-taking behavior in the laboratory are becoming clear. In human subjects studied under controlled laboratory conditions, reliable evidence that nicotine, by itself, can serve as an effective reinforcer of drug-taking behavior has until recently been primarily indirect. For example, cigarette smoke intake varies as a function of various manipulations affecting nicotine exposure, and pure nicotine medications (nicotine replacement therapy through patch, gum, nasal spray, or inhaler) can be used as temporary or long term substitutes to facilitate smoking cessation (Fiore 2000; Le Foll and George 2007). However, the persistent use of nicotine replacement therapy (NRT) provides only indirect evidence for the reinforcing effects of nicotine in humans, since NRT use may be maintained by the knowledge of the subjects that it helps smoking cessation outcome. Nevertheless, in this situation, smokers will self-administer nicotine spray more than placebo over several days after quitting smoking (Perkins 2004). However, the reinforcing effects of nicotine gum in smokers are highly dependent on instructions given to them, suggesting either that pharmacological effects are not the only factors involved in the maintenance of use of NRT (Hughes 1989) or that instructions may affect the ability of the subject to derive the pharmacological effects from the gum.

An analysis of laboratory experiments evaluating self-administration of nicotine by intravenous injection or by nasal spray in human cigarette smokers concluded that clear differences between voluntary responding for nicotine injections and saline injections had not yet been demonstrated (Dar and Frenk 2004), although these conclusions have been disputed (Perkins 2004) and recent studies now clearly indicate that human smokers will self-administer nicotine intravenously (Harvey et al. 2004; Sofuoglu et al. 2007). In a recent study conducted with male cigarette smokers who had been smoking an average of 1.5 pack of cigarettes per day for an average of 13.4 years, nicotine was shown to act as an effective reinforcer of intravenous self-administration behavior (Harvey et al. 2004) (Figs. 2e–f). Before each session, a catheter was inserted in a forearm vein for delivery of nicotine or saline. During experimental sessions, subjects sat in a chair in a test room facing a test panel with two levers and a stimulus light over each lever. When the subject pulled either lever, there was an audible click and a response was recorded. Pulling one lever repeatedly produced intravenous injections of nicotine, while pulling the other lever produced injections of saline. Note that each delivery of nicotine was associated with the presentation of a stimulus light. The number of lever-pull responses required to produce an injection varied between sessions from ten to as high as 1600. As the response requirement increased, response rates on the nicotine lever increased substantially, while rates on the saline lever remained low (Figs. 2d and f). The number of injections per session was markedly and significantly greater for nicotine than saline (Fig. 2e) and varied as a decreasing function of the dose of nicotine (Harvey et al. 2004). In these experiments, subjects adjusted their responding to increasing response requirements in a way that maintained relatively constant levels of nicotine injections per session. In another recent study, several doses of nicotine were preferred over placebo in a pure nicotine intravenous self-administration study in male and female cigarette smokers (Sofuoglu et al. 2007). The findings from these two studies clearly demonstrate that nicotine, by itself, in the absence of other constituents of tobacco smoke, can serve as an effective reinforcer of intravenous drug-taking behavior in human cigarette smokers.

The earlier difficulties in obtaining reliable intravenous nicotine self-administration and nicotine-induced conditioned place preferences across species and laboratories suggest that the reinforcing effects of nicotine, by itself, may be lower than the reinforcing effects of other drugs of abuse under many experimental conditions. These findings contrast with the apparently high reinforcing effects of tobacco smoke in human smokers. These discrepancies could be explained in part by different reinforcing effects of nicotine between species or by the influence of nonnicotine stimuli associated with smoking. An additional possibility is that the reinforcing properties of nicotine in tobacco smoke may be enhanced by other constituents of tobacco smoke. Recently, it has been shown that behavioral sensitization to nicotine, which has been implicated in drug dependence (Robinson and Berridge 1993, 2001), becomes long-lasting when nicotine is administered after treatment with a monoamine-oxidase (MAO) inhibitor (Villegier et al. 2003). Tobacco smoke is known to contain many compounds, some of which are MAO inhibitors (Fowler et al. 1996a, b). Moreover, recent results obtained

in rats suggest that treatment with MAO inhibitors may potentiate the reinforcing effects of intravenously self-administered nicotine (Guillem et al. 2005, 2006; Villegier et al. 2006). However, conflicting results have been obtained in mice (Agatsuma et al. 2006), and the results obtained in rats were obtained with a degree of MAO inhibition that is much higher than that observed in the brains of smokers (Fowler et al. 1996a, b). Further studies are needed in nonhuman primates and human subjects to validate those findings.

Another substance that is inhaled in cigarette smoke along with nicotine is acetaldehyde. Potentiation of the effects of nicotine by acetaldehyde has also been demonstrated in rodents (Belluzzi et al. 2004b), although it is unclear how this substance diffuses into the brain of smokers and how it interacts in vivo with brain reward circuitry. Further experiments are needed to clarify the role of these constituents of tobacco smoke in the reinforcing effects of tobacco.

3.3 Subjective Effects of Nicotine in Humans and Discriminative and Aversive Effects in Animals

3.3.1 Discriminative-Stimulus Effects of Nicotine in Experimental Animals

The discriminative-stimulus effects of nicotine, which are extensively used as an animal correlate of subjective reports of nicotine effects in humans, are mainly mediated by neuronal nicotinic acetylcholine receptors (nAChR), since discrimination of nicotine can be blocked by mecamylamine, a nicotinic receptor antagonist that penetrates the blood–brain barrier, but not by the nicotinic receptor antagonist hexamethonium, which does not readily enter the brain (Kumar et al. 1987; Pratt et al. 1983; Stolerman 1999; Stolerman et al. 1984). These discriminative effects are mainly mediated by high affinity nicotinic receptors (Shoaib et al. 2002; Stolerman et al. 1997). Nevertheless, a dopaminergic component may also be involved (Corrigall and Coen 1994; Desai et al. 2003; Gasior et al. 1999; Le Foll et al. 2005c). The areas of the brain that appear to be most strongly implicated in the mediation of nicotine's discriminative stimulus effects are the prefrontal cortex and the ventral striatum, but the hippocampus may also be involved (Ando et al. 1993; Miyata et al. 1999; 2002; Rosecrans and Meltzer 1981). It should be noted that the discriminative-stimulus effects of nicotine may not be related to the properties of nicotine that lead to nicotine self-administration and dependence, as suggested for other psychostimulant drugs (Spealman et al. 1999).

3.3.2 Aversive Effects of Nicotine in Experimental Animals

It has long been known that nicotine can produce both reinforcing and aversive effects, sometimes at the same dose, depending on the experimental conditions and the subject's history (Goldberg et al. 1983; Henningfield and Golberg 1983a, b).

In agreement, the same dose of nicotine may produce either positive or aversive motivational effects in rats using the place conditioning procedure (Laviolette and Van Der Kooy 2003; Le Foll and Goldberg 2005d). Similarly, squirrel monkeys will learn to repeatedly press a lever in order to obtain intravenous injections of nicotine (Fig. 3b) (Goldberg et al. 1981b). However, ongoing lever-press responding for food is completely suppressed (punished) when lever presses produce intravenous injections of the same dose of nicotine that can maintain self-administration behavior under other conditions (Fig. 3b) (Goldberg and Spealman 1983). Further, monkeys will learn to press a lever to avoid programmed injections of nicotine (Spealman 1983). Aversive effects of nicotine have also been demonstrated in rats using the conditioned taste aversion procedure with systemic nicotine injections (Reavill et al. 1986; Shoaib and Stolerman 1995; Stolerman 1988) and with intracranial infusions of nicotine (Laviolette and Van Der Kooy 2003; Shoaib and Stolerman 1995).

3.3.3 Discriminative-Stimulus and Aversive Effects of Nicotine in Humans

Human subjects can be trained to discriminate the effects of inhaled nicotine administered by nasal spray (Perkins et al. 1997) (Fig 3c). Interestingly, subjects reported both positive and negative effects following intravenous nicotine self-administration, although the positive effects were more pronounced (Fig. 2e) (Harvey et al. 2004). A recent review of the literature on subjective effects of nicotine in human subjects indicated that, across various delivery forms, nicotine increased ratings of positive effects in smokers, such as high, liking, and euphoria (Kalman 2002). Studies involving intravenous nicotine administration have reported similar positive effects, but have also shown that nicotine can elicit concurrent reports of negative effects, such as tension, jitteriness, and dysphoria (Garrett and Griffiths 2001; Henningfield et al. 1985; Jones et al. 1999; Soria et al. 1996). It is likely that subjective effects and reinforcing effects of drugs of abuse can be dissociated and that drugs of abuse may function as highly effective reinforcers even when they produce measurable reports of negative effects (Ettenberg and Geist 1991). Also, drugs of abuse may continue to function as highly effective reinforcers when dose is reduced to the point that reports of positive effects are absent (Lamb et al. 1991; Panlilio et al. 2005). Interestingly, it appears that discrimination procedures in animals and humans often provide similar results (Fig. 3). As an example, recent findings indicate that ethanol does not produce nicotine-like effects in rats (Le Foll and Goldberg 2005c), as shown in humans (Perkins et al. 2005).

3.3.4 Nicotine Withdrawal Signs in Experimental Animals

A wide range of behavioral signs (e.g., teeth chattering, chewing, gasping, writhing, head shakes, body shakes, tremors) have been noted upon cessation of chronic nicotine exposure in experimental animals (Epping-Jordan et al. 1998; Isola et al. 1999; Malin et al. 1992; Paterson and Markou 2004; Suzuki et al. 1996). Generally, rats

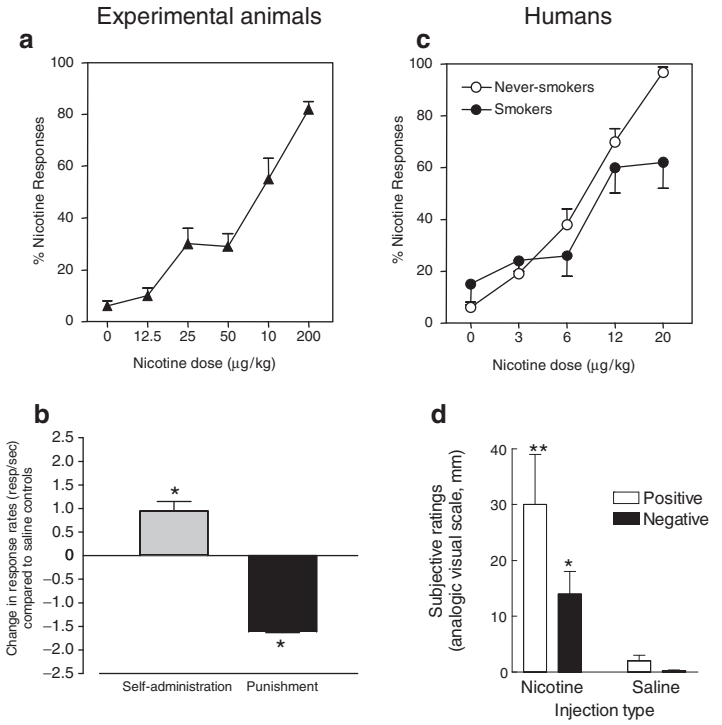

Fig. 3 Subjective and discriminative stimulus effects of nicotine in experimental animals (**a, b**) and humans (**c, d**). **a** Dose–effect functions for the discriminative-stimulus effects of nicotine in rats ($n = 8$) trained to discriminate 200 μg kg^{-1} of subcutaneously administered nicotine from saline. The percentage of responses on the lever associated with nicotine administration is shown as a function of dose (μg kg^{-1}) during tests with various nicotine doses. Adapted from Chance et al. (1977). **b** In squirrel monkeys, intravenous injections of nicotine (10.5 μg kg^{-1}) maintain intravenous self-administration behavior (adapted from Goldberg et al. 1981b), but also act like a punisher to suppress food-maintained behavior (adapted from Goldberg and Spealman 1983). **c** Dose–effect functions for the discriminative-stimulus effects of nicotine in humans (smokers or nonsmokers) trained to discriminate 20 μg kg^{-1} nicotine administered by nasal spray from placebo spray. From Perkins et al. (1997). **d** Reported positive and negative effects of nicotine injections in human subjects. Mean (+SEM) ratings of positive or negative effects (in mm) after intravenous injection of nicotine or saline on a 100 mm visual analog scale (VAS). * negative ratings were significantly greater for nicotine than saline. ** positive ratings for nicotine were significantly greater than negative ratings for nicotine and significantly greater than positive ratings for saline. *, ** $P < 0.05$, from Harvey et al. (2004)

or mice are chronically implanted with minipumps that deliver nicotine continuously. Withdrawal signs are seen after either removal of the pump or injection of a nicotinic antagonist (Malin et al. 1992; Watkins et al. 2000). To monitor physical signs of withdrawal, the number of occurrences of each sign is counted and the subject's overall withdrawal score is the number of signs cumulated across all categories (Malin et al. 1992). These behavioral withdrawal signs have been termed somatic abstinence signs or somatic behavioral signs.

The physical signs of nicotine withdrawal are often accompanied by behavioral disturbances, such as higher electrical thresholds for intracranial self-stimulation (ICSS), suggesting hypoactivity of brain reward pathways (Epping-Jordan et al. 1998). Interestingly, with mild nicotine withdrawal, indications of emotional disturbance are more likely to appear than are the behavioral somatic signs listed above. Nicotine withdrawal is also associated with avoidance behavior. Rats will avoid a compartment associated with mecamylamine-precipitated nicotine abstinence using a conditioned place preference procedure (Suzuki et al. 1996). Nicotine also has antidepressant-like effects in the forced-swim test (Tizabi et al. 1999, 2000) in Flinders-sensitive rats, a strain of rat that has been proposed as an animal model of depression (Overstreet 1995; Overstreet et al. 1995). The available evidence suggests that different underlying neurochemical deficits mediate somatic and affective components of nicotine withdrawal (see Kenny and Markou 2001 for a review).

3.3.5 Nicotine-Withdrawal Signs and Symptoms in Humans

Tobacco withdrawal induces a wide range of signs and symptoms in human smokers (Hughes et al. 1991; Hughes and Hatsukami 1986). For tobacco users trying to quit, symptoms of withdrawal from nicotine are unpleasant and stressful, but temporary. Since nicotine replacement therapy strongly decreases the intensity of withdrawal symptoms (Hughes et al. 1984; West et al. 1984a), it is assumed that the decrease in nicotine levels is responsible for the tobacco withdrawal symptoms in humans. Reducing the nicotine content of cigarettes can also result in a withdrawal syndrome (West et al. 1984b), as well as ceasing the use of nicotine gum (Hughes et al. 1986; West and Russell 1985). Signs and symptoms of nicotine withdrawal include any or all of the following: headache, nausea, constipation or diarrhea, falling heart rate and blood pressure, fatigue, drowsiness and insomnia, irritability, difficulty concentrating, anxiety, depression, increased hunger and caloric intake, increased pleasantness of the taste of sweets, and tobacco cravings. Most withdrawal signs and symptoms peak 48 h after quitting tobacco smoking and are completely gone in 6 months (Le Foll et al. 2005b). Slowing of heart rate and weight gain are distinguishing features of tobacco withdrawal, compared to other drugs of abuse (Hughes et al. 1994).

Interestingly, cessation of tobacco use increases the risk of depression (Glassman et al. 1990) and this vulnerability persists for several months (Glassman et al. 2001). However, it is unclear if this effect reflects an increased risk of depression or a relapse to depression. There is some evidence that nicotine itself may possess antidepressant properties in humans (Salin-Pascual and Drucker-Colin 1998; Salin-Pascual et al. 1996; see Picciotto et al. 2002 for a review), but these results have not yet been validated in placebo-controlled clinical trials (Thorsteinsson et al. 2001). Also, tobacco smoke contains chemical substances other than nicotine that may have antidepressant effects, possibly through the prolonged inhibition of monoamine oxidase A and B in the brain (Berlin and Anthenelli 2001; Fowler et al. 1996a, b; Meyer et al. 2006). The increased risk of depression following smoking cessation may be

related to factors other than nicotine. Nevertheless, withdrawal symptoms that occur following smoking cessation may contribute to the difficulties in quitting smoking.

3.4 Relapse Models: Influence of Stress, Drug Priming, and Presentation of Cues

3.4.1 'Relapse' in Experimental Animals

The animal model most frequently used to study relapse phenomena is reinstatement of extinguished drug self-administration behavior. See Epstein and Preston (2003), Katz and Higgins (2003) Shaham et al. (2003) for reviews and discussions on the limitations of the reinstatement model in animals for studying relapse in humans. Only limited research has been conducted with nicotine, as compared to other drugs of abuse. Various factors thought to trigger relapse in humans appear able to reinstate nicotine-seeking in laboratory animals. Studies in rats have shown that noncontingent administration of nicotine during extinction of nicotine self-administration behavior reinstates responding previously reinforced by nicotine (Andreoli et al. 2003; Chiamulera et al. 1996; Dravolina et al. 2007; Lindblom et al. 2002; Shaham et al. 1997; Le Foll et al., unpublished studies). However, the effect of nicotine priming is weak in some studies as compared to other drugs of abuse (Erb et al. 1996; Shaham et al. 1996) and effects are not found consistently (Lesage et al. 2004). Exposure to drug-paired stimuli also appears effective in reinstating extinguished nicotine-seeking behavior (Dravolina et al. 2007; Lesage et al. 2004; Liu et al. 2006; Liu et al. 2007) and in facilitating the reacquisition of nicotine self-administration behavior after a period of extinction (Caggiula et al. 2001). However, some investigators have found no effect of exposure to nicotine-paired stimuli on nicotine-seeking behavior (Andreoli et al. 2003). Exposure to stressors is also able to reinstate extinguished nicotine-seeking behavior (Buczek et al. 1999). Although all of these experiments are not entirely consistent (see above), it appears that extinguished nicotine-seeking behavior generally can be reinstated by all the factors that are effective in reinstating extinguished cocaine- or heroin-seeking behavior.

The existing treatments available to treat human smokers (Fiore et al. 2000; Le Foll and Goldberg 2007) have only recently been evaluated in animal models of nicotine dependence. The major findings are listed in Table 1. This table also reports the results obtained with drugs that have been tested both in animals and humans (for more extensive reviews see Cryan et al. 2003a, b; George and O'Malley 2004). It appears that nicotine replacement therapy (LeSage et al. 2003, 2002) and bupropion (Bruijnzeel and Markou 2003; Rauhut et al. 2003; Shoaib et al. 2003) are able to affect nicotine self-administration behavior, but the results have not been consistent across studies with bupropion (perhaps due to the role of bupropion metabolites in the therapeutic efficacy of this drug). Nicotine replacement therapy and bupropion are also effective in attenuating nicotine withdrawal signs and symptoms. These drugs have not been evaluated in animal models of nicotine relapse. Varenicline

(a nicotinic receptor partial agonist) is also an efficacious agent for treating tobacco dependence (Gonzales et al. 2006; Jorenby et al. 2006; Nides et al. 2006; Oncken et al. 2006; Tonstad et al. 2006) and it produces some effects on nicotine discrimination and on nicotine self-administration (Rollema et al. 2007). Recent evidence suggests that innovative approaches such as the blockade of cannabinoid CB_1 receptors (Cohen et al. 2005; Forget et al. 2005; Le Foll and Goldberg 2004, 2005a) or blockade of dopamine D_3 receptors (Andreoli et al. 2003; Le Foll et al. 2005a, 2003a, b), which are over-expressed in the brain of nicotine-treated animals (Le Foll et al. 2003a, b), decreases the influence of nicotine-associated stimuli or nicotine priming on nicotine-seeking behavior (Le Foll and Goldberg 2005a; Le Foll et al. 2007a).

3.4.2 Relapse in Humans

Tobacco-seeking, craving, and relapse in humans are well known to be triggered by environmental stimuli, or 'cues', that have acquired motivational salience through repeated associations with self-administered nicotine (O'Brien 2003; Shiffman et al. 2000a, b, 1986), but may also be triggered by withdrawal symptoms and tobacco smoking in abstinent subjects. Nicotine replacement therapy, bupropion, and varenicline, the three medications currently available for smoking cessation, are effective in increasing smoking cessation rates (i.e., decreased relapse rates) and are partly effective in reducing reports of craving for cigarettes in abstinent smokers (Jorenby 2002). Nicotine replacement therapy, bupropion, and varenicline may act by attenuating tobacco withdrawal symptoms (Coe et al. 2005; Shiffman et al. 2000a, b) (Table 1). Varenicline, the newest medication approved for the treatment of smokers (Cahill et al. 2007) is a nicotinic receptor partial agonist (Cahill et al. 2007). Through its intrinsic partial activation of $\alpha_4\beta_2$ nicotinic acetylcholine receptors, it may counteract withdrawal symptoms during smoking cessation attempts. Additionally, by competitively binding to $\alpha_4\beta_2$ nicotinic acetylcholine receptors, it may shield the smoker from nicotine-induced dopaminergic activation in the event that they smoke. Thus, varenicline may disrupt the reinforcing effects of tobacco and reduce nicotine withdrawal symptoms. Although this medication is efficacious in preventing smoking relapse (Tonstad et al. 2006), its effects on cue-reactivity have not yet been assessed. Continuous nicotine replacement therapy by skin patches seems relatively ineffective in attenuating reports of craving produced by smoking-associated stimuli (cues) in smokers (Tiffany et al. 2000; Waters et al. 2004). Interestingly, nicotine gum has recently been shown to be efficacious in reducing cue-induced craving for cigarettes (Shiffman et al. 2003). These different effects of nicotine patches and gum may be due either to the tolerance that occurs with continuous exposure to nicotine through skin patches or to the failure to specifically evaluate effects of the skin patches in the subgroup of subjects displaying a high degree of cue-reactivity. Recent imaging studies suggest that reports of craving and brain activation induced by environmental stimuli (cues) associated with tobacco smoking, are related to limbic brain areas (Brody et al. 2002; Due et al. 2002)

Table 1 Summary of effects of different drugs that have been tested both in experimental animals and in human smokers

Experimental animals		Human subjects	
Nicotine			
Continuous nicotine infusion decreases intravenous nicotine self-administration in rats with 23 h per day access to nicotine (no effect on cocaine self-administration).	(LeSage et al. 2003, 2002)	Increased rates of smoking cessation in controlled clinical trials using nicotine replacement therapy	(Fiore et al. 2000; Le Foll et al. 2005b; Silagy et al. 2000)
Repeated nicotine administration decreases discriminative stimulus effect of nicotine in rats.	(Robinson et al. 2006)	Nicotine exposure attenuates discriminative effects of nicotine in women.	(Perkins et al. 2001)
Nicotine withdrawal after chronic exposure through osmotic pumps or intravenous self-administration produces withdrawal symptoms.	(Epping-Jordan et al. 1998; Malin et al. 1992; Paterson and Markou 2004)	Nicotine replacement therapy attenuates nicotine withdrawal symptoms.	(Hughes et al. 1984; West et al. 1984a),
Bupropion			
Bupropion at low doses (10–30 mg kg^{-1}) had no effect on increased intravenous self-administration of nicotine, whereas higher dose of bupropion (30–78 mg kg^{-1}) decreased nicotine self-administration under a fixed-ratio schedule. Bupropion (20–40 mg kg^{-1}) had no effect on intravenous self-administration of nicotine under a progressive-ratio schedule.	(Bruijnzeel and Markou 2003; Rauhut et al. 2003; Shoaib et al. 2003)	Bupropion increased smoking cessation rate in controlled clinical trials	(Fiore et al. 2000; Le Foll et al. 2005b; Tashkin et al. 2001)

(Continued)

Table 1 (continued)

Experimental animals		Human subjects	
Bupropion produced nicotine-like discriminative effects in two out of three studies.	(Shoaib et al. 2003; Wiley et al. 2002; Young and Glennon 2002).	Bupropion decreased reports of craving and reactivity to nicotine associated stimuli. withdrawal symptoms	(Brody et al. 2004; Hurt et al. 1997; Jorenby et al. 1999; Shiffman et al. 2000a)
Bupropion enhances brain reward function and reverses the affective and somatic aspects of nicotine withdrawal in the rat.	(Cryan et al. 2003a)	Bupropion decreased tobacco withdrawal symptoms	(Brody et al. 2004; Hurt et al. 1997; Jorenby et al. 1999; Shiffman et al. 2000a)
Rimonabant (cannabinoid CB_1 antagonist)			
Rimonabant decreased intravenous self-administration of nicotine under a fixed-ratio schedule; decreased responding maintained by nicotine-associated stimuli; and blocked nicotine-induced conditioned place preference in rats.	(Cohen et al. 2005, 2002; Le Foll and Goldberg 2004; Forget et al. 2006, 2005)	Rimonanbant increased rate of smoking cessation in the STRATUS-US trial.	(Anthenelli and Despres 2004)
Rimonabant did not produce nicotine-like discriminative-stimulus effects and did not alter the discriminative-stimulus effects of nicotine.	(Cohen et al. 2002; Le Foll and Goldberg 2004)	Subjective effects of rimonabant are rated identical to those of placebo	(Huestis et al. 2001)
Varenicline (a nicotinic receptor partial agonist)			
Varenicline blocks the discriminative- stimulus effect of nicotine and the dopamine-releasing effect of nicotine. Varenicline reduces nicotine self-administration in rats and supports lower self-administration break points than nicotine.	(Rollema et al. 2007)	Varenicline increases smoking cessation rates in smoking cessation trials and appear to prevent relapse.	(Gonzales et al. 2006; Jorenby et al. 2006; Nides et al. 2006; Oncken et al. 2006; Tonstad et al. 2006)

Mecamylamine (a nicotinic receptor antagonist)			
Mecamylamine decreased intravenous nicotine self-administration under various procedures in rats and monkeys	(Goldberg et al. 1981b; Corrigal and Coen 1989; Donny et al. 1999)	Although acute mecamylamine increases nicotine self-administration and smoking, prolonged mecamylamine treatment has shown some interest for smoking cessation.	(Pomerleau et al. 1987; Rose et al. 2001, 2003a)
Mecamylamine blocks the discriminative stimulus effect of nicotine in rats	(Stolerman et al. 1997)	Mecamylamine blocks the discriminative stimulus effect of nicotine in humans	(Perkins et al. 1999; Rose et al. 1989)
Mecamylamine produces withdrawal symptoms in rats chronically exposed to nicotine	(Epping-Jordan et al. 1998; Malin et al. 1992; Paterson and Markou 2004)	Mecamylamine produces some withdrawal symptoms in smokers	(Rose et al. 2001)

and are reduced by bupropion (Brody et al. 2004). Rimonabant also seems effective in preventing relapse to tobacco use in abstinent smokers (Anthenelli and Despres 2004) (Table 1). Although Rimonabant appears to decrease the reactivity to nicotine-associated stimuli in animals, parallel experiments have not yet been conducted in humans.

4 Conclusions

In conclusion, nicotine functions as an effective reinforcer of drug-seeking and drug-taking in both humans and experimental animals. In intravenous drug self-administration studies, nicotine can serve as a prototypical drug of abuse under certain conditions, maintaining very high levels of operant responding that are clearly distinguishable from the responding maintained by saline placebo in both experimental animals and human smokers. Nicotine is also able to induce significant CPP in rodents. Thus, the reinforcing effects of nicotine have now been clearly demonstrated across procedures and across different experimental species. These procedures have revealed that nicotinic acetylcholine receptors, containing not only the α_4 and the β_2 subunits (Picciotto et al. 1998; Tapper et al. 2004), but also cannabinoid, glutamate and γ-aminobutyric acid receptors, are involved in nicotine dependence processes (Le Foll and Goldberg 2005a; Liechti et al. 2007; Paterson et al. 2004) (see also chapters from Balfour and Collins et al., this volume). Analysis of the discriminative effects of nicotine in experimental animals and reports of the subjective effects of nicotine in humans reveal a complex global effect with both positive and negative components. Both the positive and negative effects of nicotine are affected by environmental conditions and the context of the experiments, factors that may explain the difficulties in obtaining reliable results with nicotine in the past.

As with other drugs of abuse, cessation of nicotine exposure induces a withdrawal syndrome that is associated with both physical and emotional signs and symptoms. Nicotine usage may be continued by some subjects to prevent or relieve these withdrawal symptoms and, perhaps, also to prevent depression that may occur following smoking cessation. As with other drugs of abuse, nicotine priming and exposure to nicotine-associated stimuli or stressors produce reinstatement or relapse, both in experimental animals and humans. Medications that are effective in humans for increasing smoking cessation rates generally appear effective in reducing intravenous nicotine self-administration, nicotine withdrawal signs, and the effects on behavior of presentating nicotine-associated environmental stimuli, demonstrating again a strong analogy between responding of experimental animals and humans. All of these findings indicate that nicotine can act like a typical drug of abuse both in animals and humans. In addition, innovative pharmacological treatment approaches, such as the use of dopamine D_3 antagonists (Le Foll et al. 2005a, c, 2000; Pak et al. 2006) or cannabinoid CB_1 antagonists (Cohen et al. 2005; Forget et al. 2005; Le Foll and Goldberg 2005a), are under development and show promise of being able to selectively block the relapse phenomenon.

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Discriminative Stimulus Effects of Nicotine in Humans

Kenneth A. Perkins

Contents

Abstract Behavioral discrimination procedures clearly demonstrate that nicotine elicits interoceptive stimulus effects in humans that are malleable by various pharmacological manipulations as well as by some behavioral manipulations. The parameters of nicotine discrimination and both chronic and acute factors that may alter discrimination behavior are addressed in this chapter, which emphasizes research by the author involving nicotine delivered by nasal spray. Human discrimination of nicotine is centrally mediated, as the central and peripheral nicotine antagonist mecamylamine blocks discrimination but the peripheral antagonist trimethaphan does not. The threshold dose for discrimination of nicotine via spray appears to be very low in smokers as well as nonsmokers. Because smoked tobacco delivers nicotine more rapidly than spray, the threshold dose of nicotine via smoking is probably even lower. In terms of individual differences, smokers may become tolerant to the discriminative stimulus effects of higher nicotine doses but not of low doses.

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Men may be more sensitive than women to nicotine's discriminative stimulus effects, consistent with other research suggesting that nicotine is more reinforcing in men than in women. Other potential individual differences in nicotine discrimination have not been clearly tested, but may include genetics, obesity, and dependence on other drugs. Acute environmental factors that alter nicotine discrimination include the specific training and testing conditions, pointing to the need for careful control over such conditions during research. Other factors, such as concurrent acute use of alcohol or caffeine, do not appear to alter nicotine discrimination, suggesting that changes in nicotine discrimination are not likely explanations for the association of smoking behavior with use of those drugs. Concurrent physical activity also does not appear to alter nicotine discrimination, indicating that results from studies of discrimination in subjects at quiet rest, the standard approach in this research, generalize well to discrimination in subjects engaged in various activities, as often occurs in the natural environment. Future research should more clearly examine the potential role of nicotine's discriminative stimulus effects in nicotine reinforcement and determine the generalizability of these findings to nicotine delivered by other means, particularly tobacco smoking.

1 Introduction

As with most substances of abuse, nicotine produces interoceptive stimulus effects in the brain, which may be relevant to understanding its reinforcing efficacy (Holtzman 1990; Rose and Corrigall 1997; Stolerman and Jarvis 1995). Interoceptive drug effects are believed to be related to, but not synonymous with, subjective effects of drugs (Preston and Bigelow 1991). Subjective effects can include various mood changes (such as aroused, relaxed, jittery) and effects more specific to drug intake ("head rush", "euphoria") and are assessed in humans with various self-report measures, usually paper-and-pencil questionnaires (Fischman and Foltin 1991). However, the relationship between subjective reports and interoceptive stimulus effects is unclear (Preston and Bigelow 1991), and interoceptive drug effects may be only imprecisely characterized if assessed solely with self-report measures. Moreover, many self-report measures often are insensitive at low doses (Lamb et al. 1991; Perkins et al. 1994a), require good language comprehension, use terms unfamiliar to some drug users, and are lengthy to complete. Perhaps most importantly, the effects being reported by subjects cannot be independently verified, leaving open the possibility that self-report responses are not reliable indices of actual drug stimulus effects but reflect the subject's expectations of the effects of the substance (Perkins et al. 2003) or are otherwise influenced by bias.

An alternative method of assessing interoceptive drug effects is the behavioral drug discrimination procedure, which relies on observable behavioral responses to determine whether a drug's stimulus effects have been perceived by the subject (Preston 1991; Overton 1991). Behavioral drug discrimination is widely used in animal studies (Holtzman 1990; see chapter by Stolerman, this volume) because it

is essentially the only method for the study of interoceptive drug effects in subjects lacking verbal ability. However, discrimination testing in humans is also important to understanding a drug's effects. First, drug discrimination in humans provides a means to determine the extent to which findings on drug discrimination in animal research generalize to humans (Holtzman 1990; Stolerman 1999). Second, since drug discrimination is often viewed as an animal model of subjective effects in humans, direct comparison between discriminative stimulus and subjective effects is necessary and can only be done with humans (Johanson 1991). Third, drug discrimination can provide indications as to the site of action of potential therapeutic medications to treat drug dependence in humans, including smoking cessation pharmacotherapies (Perkins et al. 1999c).

Behavioral drug discrimination procedures are not without their disadvantages. For example, drug discrimination training and testing can be time consuming, while self-report measures are usually quick and easy to learn. Drug discrimination also tends to be less sensitive than self-report to qualitative aspects of stimulus effects: in other words, what effect the subject perceives rather than whether and to what degree any effect is perceived. This latter shortcoming can be remedied to some extent by the three-choice procedure (Smith and Bickel 1999), as will be discussed.

1.1 Early Nicotine Discrimination Research in Humans

Animal drug discrimination research began in the 1950s and 1960s and included studies of nicotine (Overton 1991; see chapter by Stolerman, this volume). Human drug discrimination research began soon after and increased in the 1980s, but very little of it focused on nicotine (Kamien et al. 1993), for a few reasons. First, the importance of nicotine as a drug of abuse, or the extent to which it reinforces cigarette smoking, was not clear to many researchers until the late 1980s (Russell 1979; USDHHS 1988). Second, researchers lacked adequate methods of administering nicotine in a controlled fashion, a requirement of drug discrimination training and testing. The main method, cigarette smoking, does not allow for adequate control over dosing, owing to wide variability in puff topography (Pomerleau et al. 1989). Use of tobacco smoking also confounds discrimination based on differences in nicotine versus differences in other sensory effects of smoking, such as harshness or taste (Kallman et al. 1982). Thus, early studies showing that smokers were sensitive to differences among cigarettes varying in nicotine yield (Kallman et al. 1982; Rose 1984) could not determine that this distinction was due to differences in nicotine exposure and not in other characteristics. The development of nicotine replacement therapies (NRT), first gum but then patch and other formulations, in the 1980s provided alternative dosing methods, and studies with gum will be discussed. Yet, these methods were not without problems. Largely by design, these formulations deliver nicotine rather slowly, over many minutes or even hours, rather than in seconds as with smoking (Henningfield and Keenan 1993). Slower speed of delivery alters nicotine discrimination in animals (see chapter by Stolerman, this

volume) and may result in blunted or different interoceptive effects of nicotine in humans (Henningfield and Keenan 1993; Kalman and Smith 2005). Moreover, control over dosing is still rather poor with some NRT methods, such as gum (Benowitz et al. 1997).

For all these reasons, we developed a nicotine nasal spray procedure in the mid-1980s to conduct research on acute nicotine effects in humans (Perkins et al. 1986). After completing studies on various acute effects of nicotine, we began studies of nicotine discrimination in humans in the early 1990s (Perkins et al. 1994a). Our research with this method will be discussed extensively in this chapter. This spray is similar to, but not the same as, Nicotrol, the pharmaceutical nasal spray available by prescription for smoking cessation treatment (Schneider et al. 1996; see also Perkins et al. 2007). Nasal spray delivers nicotine more rapidly than other NRT methods, with arterial nicotine peaking in about 5 min (Gourlay and Benowitz 1997), if not as rapid as smoking, and control over dosing is reasonably good (Pomerleau et al. 1989; Schneider et al. 1996). The dose–response effects of nicotine on some subjective responses are similar between smoking and nasal spray (Perkins et al. 1994b). The spray route has its own problems, including sensory irritation in the nose, requiring the use of masking agents to mimic irritation in both active and placebo sprays. However, on balance, it has provided a useful tool for the study of nicotine discrimination and other effects in humans. Intravenous infusion may be superior in terms of speed of uptake and a reduction in (but not elimination of) peripheral sensory effects (Jones et al. 1999), but it also requires extensive medical monitoring and other practical disadvantages.

1.2 Chapter Overview

This chapter will be roughly divided into two sections. The first will characterize basic parameters of nicotine discrimination in humans, such as research on the central mediation of discrimination behavior and the minimum dose, or threshold, necessary for discrimination. The second section will describe factors that moderate nicotine discrimination behavior, including static individual differences and acute situational factors. Although the chapter will occasionally discuss the association of nicotine discrimination behavior with other effects of nicotine in humans, particularly subjective effects and nicotine self-administration, the research to be discussed will focus almost exclusively on the relatively limited number of human studies using formal behavioral drug discrimination procedures. The literature on subjective mood and other self-report effects of nicotine or smoking, although somewhat relevant, is extensive and beyond the scope of the chapter. Those effects are comprehensively discussed elsewhere (e.g., Kalman 2002; Kalman and Smith 2005; Perkins et al. 1999a).

2 Characterizing Nicotine Discrimination

2.1 Overview of Procedure

Our studies all used the same basic drug discrimination procedures, adapted from those developed by others conducting research on human discrimination of opiates and other drugs (Johanson 1991; Preston 1991). Smokers in these studies all were abstinent from smoking overnight prior to each session, to prevent acute tolerance and variable baseline levels of nicotine from influencing subsequent responses to experimenter-administered nicotine by nasal spray. Subjects were first trained to discriminate a training dose of nicotine (in saline, plus capsaicin and peppermint flavoring to mask sensory effects of nicotine) from placebo (saline plus capsaicin and peppermint) and then tested for acquisition of this discrimination (see Perkins et al. 1999b, 1994a). Different doses were contained in different spray bottles. Each dose was presented in eight separate sprays, one per nostril every 20 s, to minimize the sensory effects of spray administration. During training, the two bottles were verbally identified by the experimenter with a letter code (spray "A" or "B"), but otherwise subjects were blind to their contents. Because of the speed of uptake and clearance of nicotine, we found we could present multiple trials of training and testing of discrimination in a single session, although acute tolerance can alter discrimination on trials later in the session (Perkins et al. 1996a). Thus, in both discrimination training and testing trials, subjects were intermittently administered the two bottles in random order, 20 min apart. During the test trials following training trials, subjects were instructed to identify the letter code label of the bottle (A or B) that they just received. Each correct identification was reinforced by $1 added to their payment for participation. Those who were correct on at least 80% on a minimum of five trials continued on to subsequent days of the study, usually involving testing of the generalization of discrimination across a range of doses in conjunction with other manipulations, such as pretreatment with another drug, as will be discussed.

Generalization testing involved a two-choice quantitative procedure, in which subjects distributed ten plastic chips between two sides of a box, with one side given the same letter code as the nicotine spray ("A") and the other side given the same letter code as placebo spray ("B"). Subjects were instructed to distribute these chips on the basis of whether the spray was "more like spray 'A"' (the training dose of nicotine) "or like spray 'B"' (the placebo spray). They were told they would receive $.25 for each "correctly placed" chip, to increase motivation to conduct the task according to the interoceptive stimulus effects they were perceiving. (Subjects received the maximum possible monetary reinforcement, at the end of their study participation, since there was actually no "correct" response during generalization.) The number of chips placed in the side associated with the nicotine training dose was the measure of nicotine-appropriate responding. Occasionally, a quantal procedure was also used, in which subjects made a single, dichotomous choice, identifying the spray as like one or the other by circling the letter A or B on a form.

In our first study, we demonstrated that smokers could discriminate nicotine via nasal spray (12 $\mu g\,kg^{-1}$, presented in eight sprays of 1.5 $\mu g\,kg^{-1}$ each) from placebo (Perkins et al. 1994a). Because of the peripheral sensory effects of the nasal spray method of administration (mostly nasal irritation), we were concerned about the degree to which those exteroceptive effects may be influencing discrimination behavior; the interoceptive effects of drugs are thought to be more relevant to understanding a drug's abuse liability (Holtzman 1990). In subsequent testing, subjects able to reliably discriminate the two sprays in the usual 5 min or so after administration were then required to discriminate the sprays 10 s after administration. An interval of 10 s was soon enough to discriminate the sprays on the basis of any sensory effects but too soon to do so on the basis of the interoceptive effects of nicotine by spray, because of the time required for nicotine to reach the brain. Almost all were unable to do so, suggesting that the discrimination behavior we observed during the formal study was based on nicotine's interoceptive effects and not its exteroceptive sensory effects.

2.2 Central Mediation of Nicotine Discrimination

Yet, it still was not clear that this (or any other) nicotine discrimination behavior in humans was based on nicotine's central nervous system (CNS) effects. Such effects, particularly in brain areas associated with drug reward and reinforcement (Picciotto et al. 2000), are important to understanding how nicotine may become reinforcing in human smokers (Barrett et al. 2004). Animal research had shown that central but not peripheral nicotinic blockade attenuates nicotine discrimination (see chapter by Stolerman, this volume). Furthermore, human studies had long shown attenuation of subjective responses to tobacco smoking and changes in smoking behavior as a result of pretreatment with mecamylamine, a noncompetitive nicotine antagonist that acts both centrally and peripherally (Nemeth-Coslett et al. 1986; Pomerleau et al. 1987; Rose et al. 1989; Stolerman et al. 1973). However, no study had specifically demonstrated central mediation of behavioral discrimination of *any* drug, including nicotine, in humans. Therefore, we examined the effects of pretreatment with mecamylamine versus trimethaphan, a peripheral nicotinic antagonist only, on nicotine discrimination in smokers (Perkins et al. 1999c). We reasoned that any differences between these antagonists would reflect the central antagonism of mecamylamine.

We first conducted a small preliminary study on the effects of mecamylamine pretreatment alone to identify the optimum mecamylamine dose to use in the subsequent larger study comparing it with the fast-acting intravenous peripheral antagonist trimethaphan. The methods of both studies will be described before presenting their results. In both, our standard procedure of discrimination training and testing of 0 versus 20 $\mu g\,kg^{-1}$ nicotine by nasal spray on day 1 was followed by generalization testing across a range of nicotine doses (0, 3, 6, 12, 20 $\mu g\,kg^{-1}$) on subsequent days, under various pretreatment conditions. In the preliminary study, we tested a range

of mecamylamine pretreatment doses (0, 5, 10, 15, 20 mg p.o.) prior to generalization testing, with a different mecamylamine pretreatment dose on each day. These mecamylamine doses were administered in ascending order across days for safety purposes, to identify adverse responses at the lowest possible dose. Generalization following placebo pretreatment was retested on the last day to verify that there was no change in behavior as a function of time. In the main study, we recruited additional smokers and followed the same procedures for discrimination training on day 1, followed by generalization testing of this discrimination after pretreatment with mecamylamine (10 mg p.o.), trimethaphan (10–40 $\mu g^{-1}\,kg^{-1}\,min^{-1}$ i.v. via acute transfusion), or saline on three subsequent days, in counter-balanced order between subjects. Thus, all subjects received an oral dosing (mecamylamine on one day, placebo on two days) 2 h prior to the first generalization testing trial, and an acute intravenous infusion (saline on two days, trimethaphan on one day) 2 min prior to each generalization test trial.

After the completion of each day's generalization testing in both studies, we also examined nicotine reinforcement using a choice procedure adapted from those developed by others (see deWit 1991). Our choice procedure is sensitive to smoking status (Perkins et al. 2001d), predicts greater withdrawal and relapse risk in quitting smokers (Perkins et al. 2002), and distinguishes among individual differences in smokers (Blendy et al. 2005; Ray et al. 2006). This choice procedure involves instructing subjects to intermittently self-administer a fixed number of sprays from any combination of the nicotine (2.5 $\mu g\,kg^{-1}$ per spray) or placebo bottles (see Perkins et al. 1996b). The number of times nicotine is chosen is taken as the measure of self-administration.

In the preliminary study, any active mecamylamine dose, even 5 mg p.o., attenuated nicotine discrimination, relative to placebo pretreatment (as shown in Fig. 1), collapsed across active mecamylamine doses. Mecamylamine also attenuated some subjective effects of nicotine, as also shown in Fig. 1, including "head rush" (or "buzzed"), which we have often found to relate to nicotine-appropriate responding. In the subsequent full study comparing mecamylamine and trimethaphan effects on discrimination, discrimination of the highest dose of nicotine was significantly attenuated following mecamylamine but not trimethaphan, as also shown in Fig. 1. Similar results were observed for subjective effects of nicotine (not shown).

Regarding the nicotine self-administration results (not shown), in the preliminary study mecamylamine tended to increase nicotine spray choice 25–50%, from 23.0 following placebo to 35.7, 28.7, and 32.0 out of 48 total choices, following mecamylamine pretreatment with 5, 10, and 15 mg p.o., respectively. This finding is consistent with mecamylamine's acute effect on increasing tobacco smoking behavior, presumably in an effort to override the antagonist effects of mecamylamine (Nemeth-Coslett et al. 1986; Rose et al. 1989; Rose and Corrigall 1997). However, mean nicotine choice was 17.3, or 25% lower, following 20 mg p.o. versus placebo, possibly reflecting extinction of nicotine choice behavior (since these mecamylamine pretreatment doses were administered in ascending order across days). In the larger study, mecamylamine 10 mg p.o. similarly increased nicotine choice to

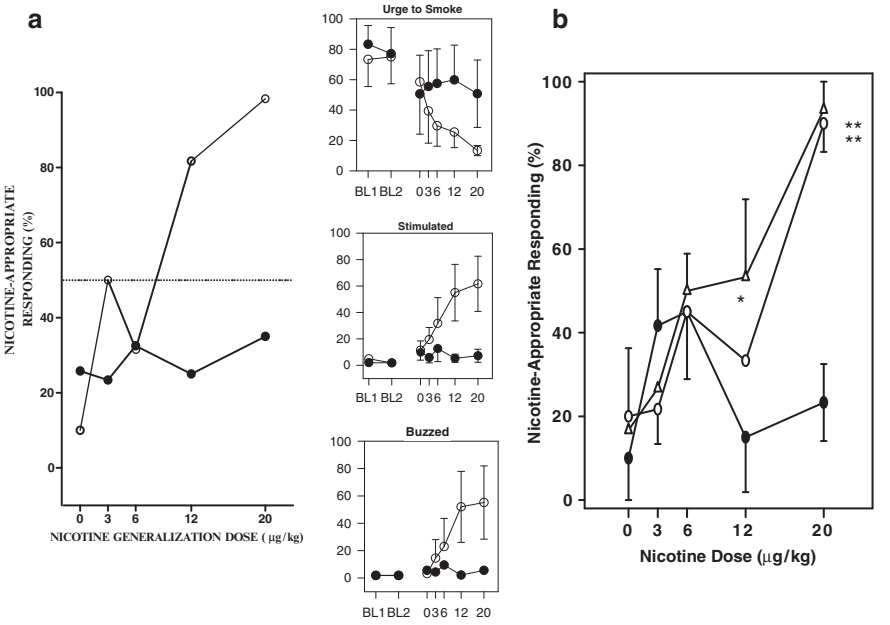

Fig. 1 **a** Mean quantitative nicotine-appropriate responding and mean ±EM selected subjective responses across nicotine generalization doses collapsed across all active mecamylamine pretreatment doses (5–20 mg; *filled circles*) and across two no mecamylamine sessions (0 mg, retest; *open circles*) in the preliminary study ($n = 3$). Subjective ratings at baselines 1 and 2 (BL1, BL2) were obtained at the beginning of each session and just before the first nicotine generalization dose trial (2 h after pretreatment), respectively. **b** Mean ±EM nicotine-appropriate responding across nicotine generalization doses as a function of pretreatment condition (oral placebo, *open circles*; 10 mg mecamylamine p.o., *filled circles*; 10–40 $\mu g\,kg^{-1}\ min^{-1}$ trimethaphan i.v., *open triangles*) ($n = 6$). $^{*}\ p < 0.05$, $^{**}\ p < 0.005$ for difference from mecamylamine pretreatment. Reprinted from Figs. 1 and 2 in Perkins et al. (1999c) with kind permission from Springer Science and Business Media

21.3, a 44% increase, from choice following placebo pretreatment, 14.8. We did not examine nicotine choice during trimethaphan administration because of practical and safety concerns.

In summary, nicotine discrimination behavior was altered by the central and peripheral nicotine antagonist mecamylamine but not by the peripheral nicotine antagonist trimethaphan, verifying that the discriminative stimulus effects of nicotine in humans are mediated centrally. To our knowledge, this was, and may remain, the first human study demonstrating central mediation of any drug's discriminative stimulus effects. One implication of this study is that drugs that antagonize nicotine centrally are likely to attenuate the discriminative stimulus effects of nicotine. To the extent that these effects are related to the reinforcing influences of tobacco smoking, such drugs may be viable candidates as novel medications to treat smoking cessation. Although side effects may preclude its eventual approval as a cessation medication, mecamylamine has shown some efficacy in promoting abstinence in

clinical trials (Rose et al. 1996). Similarly, the recent Food and Drug Administration (FDA)-approved cessation medication varenicline, a partial agonist of nicotine α4β2 receptors, among others, has been shown to blunt subjective "satisfaction" from smoking in humans (Gonzales et al. 2006), presumably due to central nicotinic activity. Whether it blunts nicotine discrimination in humans remains to be formally demonstrated but, if so, would verify that α4β2 receptors are involved in discrimination, in addition to their involvement in reinforcement (Picciotto et al. 2000; see chapter by Stolerman, this volume). On the other hand, other research has shown that trimethaphan, the peripheral nicotinic antagonist used in our study, also attenuates smoking "satisfaction" (Rose et al. 1999), pointing to the possible contribution of peripheral actions of nicotine via smoking, in addition to the central effects of nicotine.

Finally, our results with mecamylamine suggest some associations between nicotine discrimination and its subjective effects, as well as between discrimination and self-administration. Discrimination appeared to be related to the subjective effects of "stimulated", "buzzed" (or "head rush"), as well as the urge to smoke (Fig. 1), and other research also suggests that "buzzed/head rush" is linked with discrimination behavior, as will be discussed. Yet, rather than a linear association between discrimination and self-administration, as one might predict, nicotine self-administration tended to increase as a function of the attenuated discriminative stimulus effects, suggesting a relationship that is not necessarily straightforward. However, our results may be specific to acute antagonist pretreatment, and chronic blockade of nicotine's central effects could lead to reduced self-administration, as we tended to see on the last day involving mecamylamine dosing in the preliminary study.

2.3 Discrimination Threshold Dose

Aside from demonstrating that nicotine discrimination is centrally mediated, a facet of discrimination that may aid cessation treatment, as well as increase our understanding of the onset and maintenance of dependence, is identification of the lowest dose, or threshold, for nicotine discrimination. As discussed by Benowitz and Henningfield (1994), establishment by federal regulatory authorities of a maximum nicotine content in tobacco cigarettes that is very low could prevent anyone not already dependent from becoming a dependent cigarette smoker. Although extensive research is needed to identify such a maximum content, or "dose," in cigarettes, this dose is probably not lower than the threshold dose for discrimination of nicotine's interoceptive stimulus effects in nicotine-naive individuals. In other words, it is unlikely that a dose that could not be discriminated would support nicotine reinforcement (also see Lamb et al. 1991). Therefore, the lowest dose of nicotine that is reliably discriminable from placebo in nonsmokers may provide an initial estimate (or lower bound) of the threshold dose for reinforcement and the onset of dependence. The threshold dose for nicotine discrimination in dependent smokers would also be useful to verify that a cigarette dose below that discriminable by nonsmokers

is also below that discriminable by smokers. If so, then smokers with access only to these extremely low nicotine content cigarettes would not be likely to maintain dependence and, therefore, could be more likely to quit.

To determine the threshold dose for nicotine discrimination in smokers and nonsmokers, we (Perkins et al. 2001c) initially trained them to reliably discriminate our standard training dose of nasal spray nicotine ($20\,\mu g\,kg^{-1}$) from placebo on the first day. On subsequent days, we repeated this training and testing procedure across lower doses to arrive at a reliable threshold. Discrimination training and testing of only one nicotine dose from placebo occurred on each day. The threshold dose was identified as the lowest dose the subject was able to reliably (80% accuracy) discriminate from placebo on each of two different days, after failing to discriminate the next lowest dose from placebo on two days. Moreover, smokers and nonsmokers were divided into two subgroups, in which we gradually reduced (descending order) or raised from a very low training dose (ascending order) the dose to be discriminated from placebo across sessions. The purpose of varying ascending versus descending dose order was to confirm that both procedures would produce the same threshold dose estimate. For example, lower thresholds determined under the descending versus ascending order could result from a training effect over days, as progressively lower discriminable doses are administered to subjects (Rush et al. 1995; Preston and Bigelow 1998). After each dose administration, subjects completed self-report measures of mood and other effects, using the profile of mood states (POMS) and specific visual analog scale (VAS) items. We also assessed subjective "confidence" that their behavioral discrimination of the dose was correct, using a VAS item. These measures were aimed at seeing whether we might better understand the basis on which subjects discriminated threshold from subthreshold doses. Finally, on each day, after completing the trials testing acquisition of discrimination, subjects engaged in the same choice self-administration procedure as that described previously, choosing between the training doses for that day (placebo and active nicotine sprays).

Results indicated that the median threshold dose for discriminating the interoceptive stimulus effects of nicotine by nasal spray was low and similar between nonsmokers and smokers: $2\,\mu g\,kg^{-1}$ (approx. 0.14 mg for 70 kg human) and $3\,\mu g\,kg^{-1}$ (approx. 0.2 mg/70 kg), respectively, as shown in Fig. 2. Thresholds determined by descending versus ascending dose orders were comparable, increasing confidence in the reliability of these threshold estimates. The plasma nicotine levels produced by intermittent exposure (on average once every 40 min) to the threshold nicotine doses were 1.6 and $2.6\,ng\,ml^{-1}$ for nonsmokers and smokers, respectively. Self-administration did not differ between the threshold and next lowest dose (i.e., subthreshold), as smokers selected nicotine on a mean of 6.1 versus 7.4 out of 16 possible choices, respectively, and nonsmokers selected nicotine on 3.6 versus 4.7 choices, respectively. (The overall difference in choice was significantly greater in smokers versus nonsmokers.) Because eight choices represents chance (50% of 16 total choices), nonsmokers appeared to choose the nicotine spray less than placebo, even when the dose of the nicotine spray was below the discrimination threshold dose. Yet, smokers did not choose nicotine above chance levels in this study.

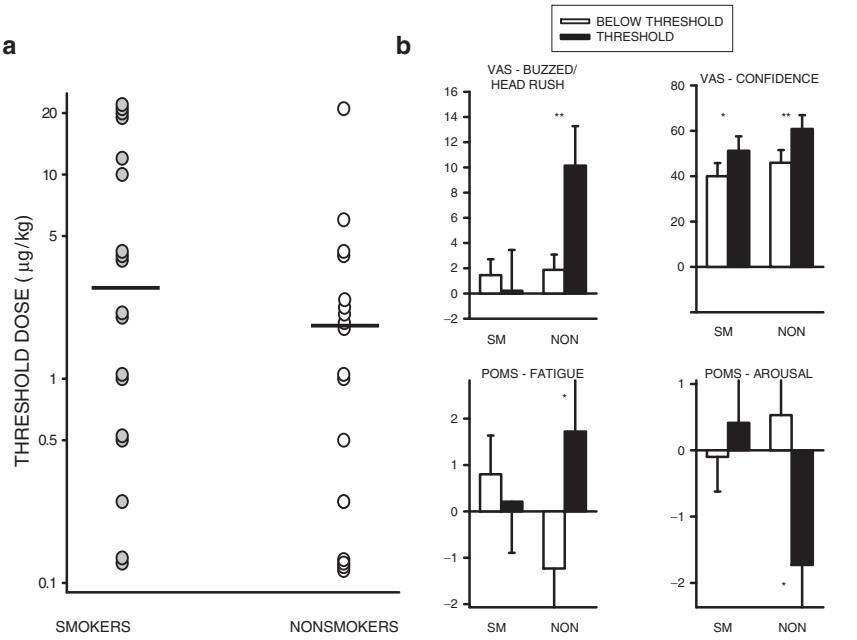

Fig. 2 **a** Distribution of threshold doses (in $\mu g\,kg^{-1}$) for nicotine nasal spray discrimination in smokers ($n = 18$) vs. nonsmokers ($n = 17$). *Horizontal lines* designate group median thresholds, which did not differ between groups. **b** Selected subjective responses to nasal spray nicotine doses at, and just below, threshold for discrimination in smokers and nonsmokers. * $p < 0.05$; ** $p < 0.01$ for difference between doses. Reprinted from Figs. 1 and 2 in Perkins et al. (2001c) with kind permission from Springer Science and Business Media

Subjective effects distinguished threshold from subthreshold doses in nonsmokers but not in smokers, as also shown in Fig. 2. Smokers may distinguish these doses on the basis of interoceptive effects related to other unassessed subjective measures, such as whatever may be driving their "confidence" ratings (see Fig. 2) or on some other interoceptive changes not easily measured by verbal methods. This observation is consistent with our finding from a different study that greater "buzzed/head rush" effects of nicotine, one of those differentiating threshold and subthreshold doses in nonsmokers here (Fig. 2), were positively associated with nicotine spray reinforcement in smokers but inversely associated in nonsmokers (Perkins et al. 2001d). As discussed later on, we also found in another study that discrimination behavior was more strongly influenced by the training dose in nonsmokers compared with smokers (Perkins et al. 1999b). Thus, despite the appearance of a similar sensitivity to nicotine discrimination between nonsmokers and smokers in Fig. 2, the processes involved may differ as a function of smoking status.

The median threshold in smokers of $3\,\mu g\,kg^{-1}$ nicotine is consistent with previous research reporting an ED_{50} for discrimination (the dose which 50% of subjects can discriminate) of $3\,\mu g\,kg^{-1}$ in smokers trained to discriminate $12\,\mu g\,kg^{-1}$ from placebo (Perkins et al. 1994a). In the current study, the mean blood level of

nicotine in smokers following intermittent exposure to the threshold dose was less than 3 ng ml^{-1}, far below the blood levels typically observed in smokers following intermittent smoking of a few cigarettes (Benowitz et al. 1994; Gourlay and Benowitz 1997). Thus, smokers likely self-administer amounts of nicotine that are well above their threshold dose for discrimination, consistent with the observation by others that the dose in most caffeinated beverages is well above the threshold dose for caffeine discrimination in some regular caffeine users (as low as 1.8 mg, a twentieth of that in soda or a fiftieth of that in brewed coffee; Mumford et al. 1994).

On the other hand, the range of threshold doses varied by over 100-fold in both groups, smokers and nonsmokers, from 0.13 up to 20 μg kg^{-1} (Fig. 2), which was the training dose on day 1 that all subjects had to be able to discriminate in order to proceed with further testing of threshold dose. This finding indicates substantial individual differences in sensitivity to the interoceptive stimulus effects of nicotine that are independent of smoking status. Whether the same degree of variability, and the association of various factors with that variability, would be seen with discrimination of nicotine via cigarette smoking remains to be determined.

The implications of this research for understanding nicotine discrimination via tobacco smoking depend on a number of factors, including the bioequivalence of nicotine exposure between smoking and nasal spray, and especially on the degree to which nicotine discrimination is influenced by the kinetics of the administration method, which also differ between smoking and spray. If our results are directly relevant to discrimination via smoking, the median threshold in nonsmokers of 2 μg kg^{-1} nicotine by spray suggests that only very modest exposure is needed for tobacco-naive individuals, such as teens, to "feel the effects" of nicotine from smoking. The level of nicotine exposure from 2 μg kg^{-1} by spray may be lower than the typical exposure from smoking in teens who smoke only a few cigarettes per day (Eissenberg and Balster 2000). The actual nicotine deliveries (rather than FTC-measured "yields") of most cigarette brands smoked in normal fashion exceed 0.4 mg per cigarette (or about 6 μg kg^{-1}; Byrd et al. 1998), equal to or greater than the threshold doses of all but one of the nonsmokers in this study. Yet, more rapid methods of drug delivery, such as smoked or intravenous infusion, produce stronger responses than slower methods (Cone 1995; Henningfield and Keenan 1993), including nasal spray as well as transdermal patch. Thus, even smaller doses of smoked nicotine may be readily discriminable by smokers and nonsmokers alike.

3 Moderators of Nicotine Discrimination

Nicotine discrimination behavior, which is mediated by nicotine actions in the CNS, is strongly related to dose, as indicated in tests of generalization across doses (e.g., Fig. 1), and may be sensitive to very small doses of nicotine (i.e., threshold), below those commonly administered via cigarette smoking. However, it would be a mistake to view any drug discrimination behavior as based solely on the intensity of the interoceptive stimulus effects of that drug. The observed discrimination behavior is

substantially a function of environmental factors, such as the testing conditions (e.g., training dose), and may vary owing to individual difference characteristics. This section will examine the influence of some static individual difference characteristics and of acute environmental conditions on moderating nicotine discrimination behavior. Relatively little research has studied these potential moderators, perhaps because of the large sample sizes needed to compare responding between subgroups differing on characteristics, and the extensive number of sessions required to test the influence of various environmental manipulations on discrimination.

3.1 Individual Differences in Sensitivity to Nicotine Discrimination

Two individual differences we have formally examined in several studies of nicotine discrimination are smoking status and subject sex. Both will be discussed here. Effects of the chronic use of other drugs will also be briefly considered.

3.1.1 Smoking Status

The study on discrimination threshold dose, described previously, generally found little difference in threshold between smokers and nonsmokers, although self-reported subjective effects seemed to differentiate threshold from subthreshold doses in nonsmokers and not in smokers. However, discrimination behavior at higher nicotine doses – closer to those commonly experienced by smokers – may reveal clearer differences in sensitivity due to smoking status. In a study by others, for example, cocaine users unable to acquire discrimination between cocaine and placebo used more of the drug per occasion and reported less intense subjective responses to the training doses, relative to those who were able to acquire the discrimination (Singha et al. 1999). Thus, greater drug use may lead to chronic tolerance to some of the drug's effects, including those relevant to discrimination. We sought to determine if greater nicotine use similarly attenuated discrimination of nicotine at doses higher than the threshold dose. One study directly comparing nicotine discrimination in smokers and nonsmokers is presented below (Perkins et al. 1997). A second study comparing the effects of smoking status on discrimination also involved a manipulation of training dose (Perkins et al. 1999b) and so is described later under environmental moderators of discrimination.

We first tested differences in nicotine discrimination as a function of smoking status in a study involving our standard procedure of training subjects to discriminate $20\,\mu g\,kg^{-1}$ versus 0 via nasal spray on day 1, followed by a test of generalization of this discrimination across a range of nasal spray nicotine doses on day 2 (Perkins et al. 1997). Both quantitative and quantal (a single, dichotomous choice) procedures were used in generalization. On day 3, we assessed nicotine self-administration via the same choice procedure as that described previously, involving intermittent choice of sprays between nicotine and placebo bottles. All ten

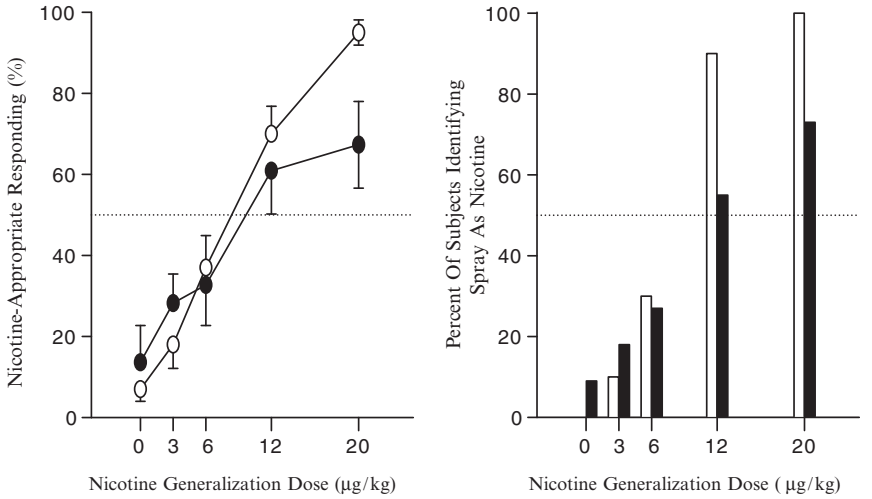

Fig. 3 Quantitative (*left*) and quantal (*right*) measures of generalization of discrimination across nicotine generalization doses in smokers (*filled circles or bars*, $n = 11$) vs. nonsmokers (*open circles or bars*, $n = 10$). *Dotted line* indicates 50% or chance responding. Group differences in responding were observed at $20\,\mu g\,kg^{-1}$ for quantitative and $12\,\mu g\,kg^{-1}$ for quantal responding. Reprinted from Fig. 1 in Perkins et al. (1997) with kind permission from Springer Science and Business Media

nonsmokers and all but one of the 11 smokers learned to discriminate the training doses, although self-reported ratings of "confidence" in the accuracy of discrimination behavior after training were significantly lower in smokers than in nonsmokers. During generalization, responding across doses significantly differed between smokers and nonsmokers (i.e., a dose by smoking status interaction). As shown in Fig. 3 (left panel), quantitative responding was lower in smokers than nonsmokers at the top dose, $20\,\mu g\,kg^{-1}$ (i.e., the training dose), but responding was not significantly different at lower doses. Quantal responses, also shown in Fig. 3 (right panel), indicated a similar reduction in nicotine-appropriate behavior at the two higher doses, 12 and $20\,\mu g\,kg^{-1}$, in smokers versus nonsmokers.

In examining potential correlates of nicotine's interoceptive effects that may help explain discrimination behavior, only the subjective response of "head rush" was related to discrimination behavior, especially in nonsmokers. Nicotine choice, assessed on day 3, was significantly greater in smokers versus nonsmokers, as expected. We also found that nicotine choice within nonsmokers was significantly and *inversely* associated with nicotine discrimination responding during generalization testing on day 2, while the association was nonsignificantly positive in smokers. Therefore, greater discrimination behavior in nonsmokers at the highest nicotine dose was related to reduced self-administration of that same dose. This observation demonstrates that, in contrast with drug self-administration, drug discrimination is "hedonically neutral" and can be associated with interoceptive stimulus effects that are aversive as well as pleasurable.

From this study, along with the study of threshold dose for discrimination in smokers and nonsmokers presented earlier, it appears that smokers are less sensitive than nonsmokers to the discriminative stimulus effects of higher nicotine doses but not to lower nicotine doses. Thus, chronic smoking may induce chronic tolerance to these (and other) effects of higher doses of nicotine but not to lower doses. If so, these findings could help explain escalation of nicotine intake with chronic use, to overcome tolerance development, but they also suggest that smokers retain sensitivity to lower nicotine doses throughout their smoking careers, from experimentation as teens to maintenance of dependence. Moreover, the top dose here, 20 $\mu g\,kg^{-1}$, is still relatively modest in that it results in nicotine blood levels less than those seen in smokers after comparable intermittent smoking (Perkins et al. 1994b). Even greater differences in discrimination due to smoking status may be seen in studies of larger nicotine doses.

Differences in nicotine discrimination as a function of other smoking histories should be studied to better determine the influence of chronic smoking exposure on discrimination. For example, nondependent smokers ("chippers") are those who smoke just a few cigarettes per day for years without becoming dependent but who also self-administer doses similar to those of dependent smokers (Perkins et al. 2001d; Shiffman et al. 1992). Differences in discrimination between nondependent and dependent smokers could clarify the relationship between discrimination and nicotine dependence (Perkins et al. 2001e). Similarly, exsmokers retain much of the tolerance to nicotine's effects seen in current smokers, despite years of abstinence (see Perkins 2002). Comparison of discrimination between current and exsmokers could determine whether or not tolerance to the discriminative stimulus effects of higher nicotine doses in current smokers (Fig. 3) persists after quitting.

3.1.2 Subject Sex

We have included men and women in all of our nicotine discrimination studies, although the sample sizes have usually been too small to allow detection of sex differences in nicotine effects. Nevertheless, in some but not all studies, we have seen that women, relative to men, tend to have more difficulty in acquiring the initial training dose discrimination, and/or show responding during generalization that tends to be flatter across doses (Perkins et al. 1997, 1996a). Thus, women appear to be less sensitive than men to the discriminative stimulus effects of nicotine, at least via nasal spray. Because this research on sex differences in nicotine discrimination was reviewed elsewhere (Perkins et al. 1999b), it does not need to be presented in detail here. Briefly, however, these sex differences in nicotine discrimination may be found only among smokers and not in nonsmokers (Perkins et al. 1997), suggesting a sex difference in the long-term adaptation to nicotine intake rather than an innate relative insensitivity in women.

The observation of sex differences in nicotine discrimination is consistent with more extensive research showing that the reinforcing and rewarding effects of nicotine are less robust in women versus men (Perkins et al. 1999a, Perkins 2008), and

that women may obtain less therapeutic benefit than men from nicotine replacement (Perkins and Scott 2008). For example, nicotine self-administration in our choice procedure is often less among women than men (Perkins et al. 1997; 2001d). On the other hand, women may be more responsive than men to the nonnicotine stimuli (e.g., cues) accompanying nicotine intake via smoking or other means (Perkins et al. 2001f, Perkins 2008). This is perhaps consistent with observations from discrimination research that women tend to respond to placebo administration with greater nicotine-appropriate behavior, indicating that they respond more to the nondrug stimulus effects than the drug stimuli (Perkins et al. 1999b). A broader explanation for these sex differences may come from research suggesting that men are more sensitive to interoceptive cues for affect (mood), while women are more responsive to exteroceptive cues (Roberts and Pennebaker 1995). Nicotine discrimination requires accurate perception of interoceptive effects, while nonnicotine stimuli of smoking provide mostly exteroceptive effects.

Future research should verify the magnitude of any sex differences in nicotine discrimination and, if reliable, examine the role of sex hormones as causal mechanisms for this sex difference. Moreover, because women may also be more responsive to nonnicotine stimuli that accompany cigarette smoking (Perkins et al. 2001f), future research should explore whether presentation of such stimuli enhances discrimination of nicotine in women. The possible influence of such stimuli on nicotine discrimination is addressed briefly near the end of this chapter.

3.1.3 Other Possible Individual Differences

The individual differences in vulnerability to the onset and/or persistence of nicotine dependence is a very active area of research interest (see Audrain-McGovern et al. 2009; Lerman et al. 2009). In particular, genetic influences on dependence vulnerability are under study. Although no study has examined genetic influences on nicotine discrimination in humans, some research has identified genetic factors in acute responses to nicotine administration that may relate to its discriminative stimulus effects. For example, Ray et al. (2006) showed that smokers with the G allele (homozygous or heterozygous) of the μ-opioid receptor gene OPRM1 reported less difference in subjective "strength" and "satisfaction" between a nicotine and denicotinized cigarette, compared with smokers homozygous for the A allele. Presumably, "strength" reflects perception of nicotine intake from smoking, although such perception could be peripheral (e.g., throat sensations) as well as central in nature (see Rose 2006). Ray et al. (2006) also found an interaction of OPRM1 allele by sex on nicotine choice using the same procedure as that described previously, only with nicotine and denicotinized cigarettes rather than nasal spray. Nicotine choice was lower for those with the G versus A allele among women but not among men. The main effect of OPRM1 on nicotine choice was marginally significant. Whether or not OPRM1 would also influence nicotine discrimination behavior remains to be seen. Nicotine discrimination as a function of other genes would be an important

future research direction. Given the wide variability in discrimination threshold dose within smokers and within nonsmokers (Fig. 2), genetic factors could be especially relevant to understanding discrimination threshold.

Aside from differences in nicotine discrimination due to chronic tolerance from long-term use of nicotine (i.e., smoking status), discussed previously, nicotine discrimination may vary owing to long-term use of other psychoactive drugs, via cross-tolerance or cross-sensitization (Desai and Terry 2003). Compared with nonusers of drugs, smoking is more prevalent among abusers of other drugs (Kozlowski et al. 1989), and chronic use of other drugs is associated with increased vulnerability to nicotine dependence (Patton et al. 2005), perhaps by altering nicotine discrimination. In the only published controlled study on this question in humans, to our knowledge, Madden et al. (1995) tested the reverse association, sensitivity to acute alcohol as a function of smoking status within a group of alcohol drinkers, and found that self-reported "intoxication" response to alcohol was attenuated in smokers versus nonsmokers. The relevance of these results to understanding the influence of chronic alcohol intake on nicotine discrimination is not clear, as the relationship between past drug use and sensitivity to another drug may not be symmetric (Desai et al. 1999). Moreover, this study could not confirm whether smoking was directly responsible for the difference in sensitivity to alcohol, as smoking status might instead have covaried with another factor (such as genetics) that might influence alcohol sensitivity.

As a third example of other possible individual differences in nicotine discrimination, sensitivity to the acute effects of nicotine may reflect a broader individual variation in psychoactive drug sensitivity and not a difference specific to nicotine's effects. That broader variability in drug sensitivity could reflect genetic or other prominent individual difference characteristics responsible for neurophysiological responses to drugs. For example, in samples of smokers who were also regular caffeine and/or alcohol drinkers, we observed that greater acute sensitivity to selected subjective and cardiovascular effects of nicotine was associated with greater acute sensitivity to the same effects of caffeine and, to a lesser extent, alcohol (Perkins et al. 2001a). Those who responded to nicotine with greater "dizzy" and "head rush", effects that have been associated with nicotine discrimination behavior (Perkins et al. 1997), also responded to caffeine or to alcohol with greater "dizzy" and "head rush". Other effects similarly increased by nicotine and caffeine included "fatigue", "pleasant", "relaxed", "comfortable", and "jittery", as well as systolic and diastolic blood pressure. Fewer effects were similarly increased by nicotine and alcohol but included "fatigue", "relaxed", and "jittery", as well as systolic blood pressure. Other research indicates that some subjective responses to nicotine administered intravenously may resemble responses to intravenous cocaine or amphetamine (Jones et al. 1999), suggesting that similar individual variability may exist between nicotine and cocaine or amphetamine effects. No human research has directly compared sensitivity to the discriminative stimulus effects of nicotine versus other drugs, but these findings suggest a possible association.

On the basis of research on subjective or reinforcing effects of acute nicotine, other individual difference characteristics may moderate nicotine discrimination, such as sensation-seeking personality in nonsmokers but not smokers (Perkins et al. 2000), or obesity status (Blendy et al. 2005).

3.2 Environmental Moderation of Nicotine Discrimination

Aside from the chronic differences due to the stable individual characteristics discussed previously, the discriminative stimulus effects of nicotine may vary acutely owing to shifting environmental factors, certainly within hours and perhaps within minutes. While nicotine's interoceptive stimulus effects certainly have neurophysiological correlates (Barrett et al. 2004), it is important to recognize that discrimination is ultimately a behavioral response that can be altered by the same factors that moderate any human behavior. Moreover, it is well known that nicotine's subjective mood and hedonic effects can vary according to environmental factors (e.g., Kalman 2002; Perkins et al. 1999a). Even the perceived nicotine content of cigarettes, which likely influences nicotine discrimination via smoking, can be by altered by instructional manipulations (Perkins et al. 2003, 2008). So, it is reasonable to expect that such factors may also moderate nicotine's discriminative stimulus effects. The environmental factors that may *potentially* alter nicotine discrimination are numerous, but three such factors will be discussed here: the specific conditions of discrimination training and generalization testing, concurrent exposure to other drugs, and intensity of concurrent physical activity engaged in while experiencing the interoceptive stimulus effects of nicotine.

3.2.1 Training and Testing Conditions

Often overlooked is the fact that discrimination responding is very strongly determined by the specific conditions of training and generalization testing. The two conditions to be discussed here are the doses used during training and the different response options during generalization testing. In most discrimination research, it is responding during generalization testing that usually is of interest, to determine differences in nicotine discrimination behavior as a function of any number of manipulations, such as individual difference characteristics, acute pretreatment condition (such as nicotine antagonist drug; Fig. 1), or generalization between nicotine and comparison drugs or novel compounds. Responding during training typically is important only to the extent that it verifies that the subject has acquired discrimination of the training dose prior to generalization testing. However, responding during generalization is a clear function of the doses used during training. After all, generalization testing involves subjects making a behavioral choice that reflects the relation between the test dose they just received and the two (or more) training doses they learned to discriminate during training. An analogous procedure may be

the "matching to sample" task, where a variation in the "sample" (i.e., the training stimulus) will alter "matching" behavior (i.e., drug-appropriate responding; van Hest and Steckler 1996).

We conducted two studies examining the influence of nicotine training dose on subsequent generalization of responding across a range of nicotine doses. In the first study (Perkins et al. 1996a), smokers were randomly assigned to a day 1 training dose of either 10 or 30 $\mu g\,kg^{-1}$ via nicotine nasal spray, to learn to discriminate from placebo. All then received the same test of generalization on day 2, involving administration of 0, 5, 10, 20, and 30 $\mu g\,kg^{-1}$ nicotine via spray in that order. (Doses were administered in ascending order to prevent acute tolerance due to large doses presented early in the session.) Nicotine-appropriate responding was shifted significantly to the left, indicating enhanced discrimination, in the group trained to discriminate 10 $\mu g\,kg^{-1}$ from placebo, compared to the group trained to discriminate 30 $\mu g\,kg^{-1}$ from placebo. Results were remarkably similar to those of research on nicotine discrimination as a function of training dose in rodents (Stolerman et al. 1984; see chapter by Stolerman, this volume).

In the second study (Perkins et al. 1999b), we manipulated within subjects not only the training dose but also the number of response options during generalization testing, to demonstrate how training and generalization testing conditions alter discrimination responding. We also included nonsmokers as well as smokers, to determine whether these influences could vary owing to chronic nicotine exposure. The manipulation of training dose involved first training subjects to discriminate our standard dose of 20 $\mu g\,kg^{-1}$ nicotine by nasal spray from placebo on day 1 and then to assess generalization across a range of doses from 0 to 20 $\mu g\,kg^{-1}$ on day 2. We then proceeded to identify each subject's threshold dose for discrimination on subsequent days (as described previously; see Fig. 2). Finally, that threshold dose and placebo were used as new training doses prior to repeat assessment of generalization across the same range of doses from 0 to 20 $\mu g\,kg^{-1}$.

The manipulation of generalization response options involved adding a three-choice quantitative procedure to our standard two-choice quantitative procedure, both of which were completed back-back during each testing trial. In the three-choice procedure, which was based on the work by Bickel and colleagues (Bickel et al. 1993; Smith and Bickel 1999), subjects were instructed to distribute the ten chips among three bins, labeled A, B, and C, with the first two representing "like spray A" and "like spray B" as in the two-choice procedure, and the last to be used if the spray was "like neither A nor B". The number of chips in the C bin was taken as "novel-appropriate" responding. This option can be important in identifying stimulus effects of drugs that are qualitatively different (i.e., novel) from the training doses, which is not possible with the two-choice procedure (Bickel et al. 1993). While the novel response option is particularly useful in testing the generalization of responding from one drug to a different drug, nicotine may have qualitatively different stimulus effects at different doses (Jones et al. 1999).

Similar to our previous study of training dose effects (Perkins et al. 1996a), generalization testing across the range of intermediate doses (0–20 $\mu g\,kg^{-1}$) with the two-choice procedure showed a shift to the left in nicotine-appropriate responding

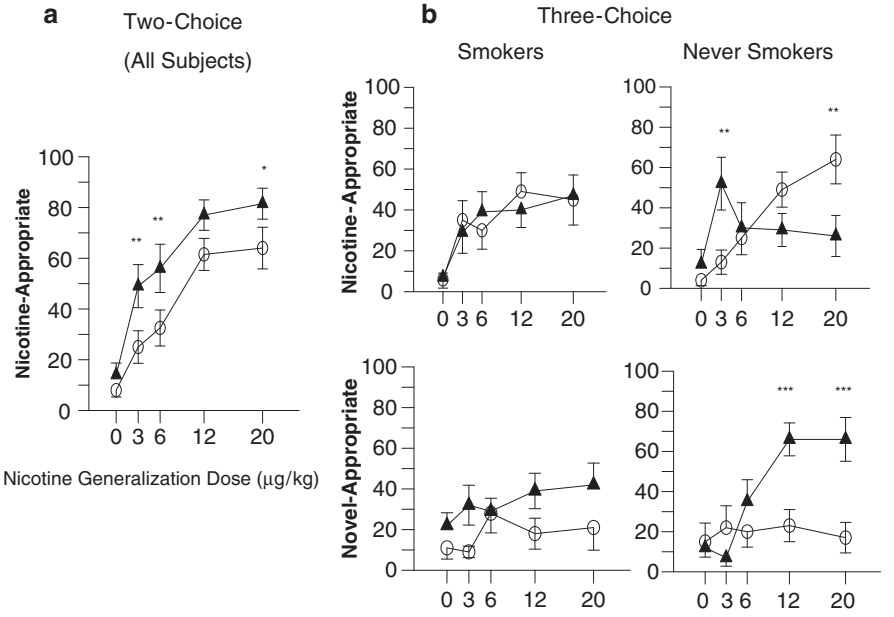

Fig. 4 **a** Nicotine-appropriate discrimination behavior across nicotine generalization doses in the two-choice quantitative procedure as a function of training dose condition ($20\,\mu g\,kg^{-1}$, *open circles*, versus subject's threshold dose, *filled triangles*) in all subjects, smokers as well as nonsmokers ($n = 10$ each), who did not differ. $^{*}p < 0.05$; $^{**}p < 0.01$ for differences between training dose conditions at specific nicotine generalization doses. **b** Nicotine- and novel-appropriate discrimination behavior across nicotine generalization doses in the three-choice quantitative procedure as a function of training dose condition in smokers versus nonsmokers. Training dose symbols as in **a**. $^{**}p < 0.01$; $^{***}p < 0.001$ for differences between training dose conditions at specific nicotine generalization doses. Reprinted from Figs. 1 and 2 in Perkins et al. (1999b) with kind permission from Springer Science and Business Media

when the threshold dose was the training dose, compared to when $20\,\mu g\,kg^{-1}$ was the training dose (as shown in Fig. 4). Mean threshold doses were similar between smokers and nonsmokers (3.5 and $1.9\,\mu g\,kg^{-1}$, respectively). Unlike our prior study comparing smokers and nonsmokers on discrimination responding, there was no effect of smoking status on generalization in the two-choice procedure. However, in the three-choice procedure both nicotine- and novel-appropriate responding were significantly influenced by training dose in nonsmokers but not in smokers. As also shown in Fig. 4, nonsmokers exhibited more nicotine-appropriate responding at low generalization doses and more novel-appropriate responding at higher generalization doses when the threshold dose was the training dose, compared to when $20\,\mu g\,kg^{-1}$ was the training dose. Interestingly, the subjective effect of "head rush" in response to generalization doses was shifted to the left when the threshold dose was the lower training dose, similar to nicotine-appropriate responding under the two-choice procedure, suggesting again that this subjective effect may be related to nicotine discrimination. Moreover, the subjective effect of "jittery" was also shifted to the left as a function of the lower training dose, but in nonsmokers only. This

effect may be related to the greater novel-appropriate responding to the higher generalization doses in nonsmokers when trained with the threshold dose (Fig. 4). In summary, these results show the strong malleability of discrimination responding, as well as subjective responses, as a function of the training or generalization testing conditions, along with individual difference characteristics (smoking status).

3.2.2 Concurrent Drug Use

Any psychoactive drug, virtually by definition, has interoceptive stimulus effects. When drugs are used together, the resulting stimulus effects may combine in additive fashion or vary in ways beyond the simple additive effects of each drug. This issue may be clinically important, given the very high prevalence of smoking among abusers of other drugs and the high rates of other drug use among smokers (Kozlowski et al. 1989). Moreover, use of some drugs, such as alcohol and stimulants (Mitchell et al. 1995; Rush et al. 2005), acutely increases the frequency of smoking, and the resulting combined stimulus effects may help explain why. The discriminative stimulus effects of nicotine in combination with other drugs has been examined extensively in rodent research (see chapter by Stolerman, this volume) but less so in human research. In separate studies with smokers, we have examined nicotine discrimination following pretreatment with (a) nicotine itself, (b) alcohol, and (c) caffeine, and those findings will be described here. In each study, we also examined nicotine self-administration via our choice procedure, under each pretreatment condition but after the completion of nicotine generalization testing.

Nicotine pretreatment

Changes in nicotine discrimination as a function of pretreatment with nicotine may be relevant to understanding why smokers typically report that the first few cigarettes of the day are the most enjoyable and have the greatest subjective effects (Fant et al. 1995). Subsequent smoking may be less enjoyable because of acute tolerance to the acute effects of nicotine intake (Perkins et al. 1995), including its discriminative stimulus effects (Perkins et al. 1996a). In addition, perhaps use of nicotine replacement therapy (NRT) when quitting, such as gum or patch, aids cessation by blunting the discriminative stimulus effects of nicotine via smoking. We examined this issue by training smokers to discriminate our standard dose of $20\,\mu g\,kg^{-1}$ nicotine by nasal spray from placebo on day 1, and then tested for generalization across a range of nicotine spray doses on subsequent days following pretreatment with placebo, moderate, or high dose nicotine by patch (Perkins et al. 2001b). Two patches were applied each day to achieve the desired placebo (two placebo patches), moderate (one placebo and one active patch of 14 or 21 mg, depending on subject's body weight), and high (two active patches) nicotine pretreatment exposure. Generalization testing across nicotine doses by nasal spray occurred 3 h after patch application, to allow time for nicotine from the patch to be absorbed.

Plasma nicotine samples showed no difference due to body weight in blood nicotine concentration following active patch pretreatment, as expected, confirming equal dosing across subjects.

Results showed no overall effect of patch pretreatment on generalization responding in the two-choice procedure, although nicotine patch pretreatment attenuated responding at intermediate spray doses in women and enhanced responding at the highest spray dose in men. Furthermore, patch pretreatment increased novel-appropriate responding in the three-choice procedure at placebo and intermediate spray doses, especially in men. Nicotine patch pretreatment tended to decrease nicotine spray choice in men (11.6, 9.5, and 7.8 nicotine choices following placebo, moderate, and high patch pretreatment, respectively, out of 24 total choices), but did not alter choice in women (7.9, 8.9, and 7.4, respectively). A dose-dependent decrease, as found in men, is expected if the regulation of nicotine intake influences self-administration. Moreover, we found sex differences in the subjective effect most associated with discrimination behavior (POMS arousal in men versus "head rush" in women), although both responses were significantly attenuated by patch pretreatment condition.

In general, these findings suggest that nicotine pretreatment by patch induces relatively little acute tolerance to the discriminative stimulus effects of nicotine administered by spray, despite tending to attenuate some subjective effects and, in men, nicotine choice. Other research shows clear acute tolerance to effects of repeated nasal spray nicotine (e.g., Perkins et al. 1995), including discrimination (Perkins et al. 1996a), suggesting that acute tolerance to nicotine discrimination may depend on the manner of the pretreatment dose administration. Conceivably, repeated generalization testing throughout the day, simulating additional cigarettes smoked later on, may have shown stronger evidence of acute tolerance, but that would likely be due to accumulation of rapid nicotine intake via spray rather than the patch pretreatment. The effect of nicotine pretreatment on nicotine discrimination should be further examined with different methods of administration, both at pretreatment and discrimination testing.

Alcohol pretreatment

Alcohol has been shown to acutely increase smoking behavior (Mitchell et al. 1995), and so the influence of concurrent alcohol consumption on nicotine discrimination could be important to understanding alcohol's effect on smoking reinforcement. We examined the effects of alcohol pretreatment on nicotine discrimination in smokers in a procedure similar to the prior study of nicotine patch pretreatment (Perkins et al. 2005a). Subjects were trained to discriminate $20\,\mu g\,kg^{-1}$ from placebo on day 1 and then tested for generalization across a range of nicotine spray doses on subsequent days following pretreatment with placebo, 0.4, or $0.8\,g\,kg^{-1}$ alcohol. Intermittent "topping" doses of alcohol were administered to maintain a steady blood alcohol level throughout the course of generalization testing. Results showed no significant effect of alcohol pretreatment on nicotine discrimination behavior, as shown in Fig. 5, or on nicotine choice (15.2, 15.1, and 16.4 choices following placebo, 0.4,

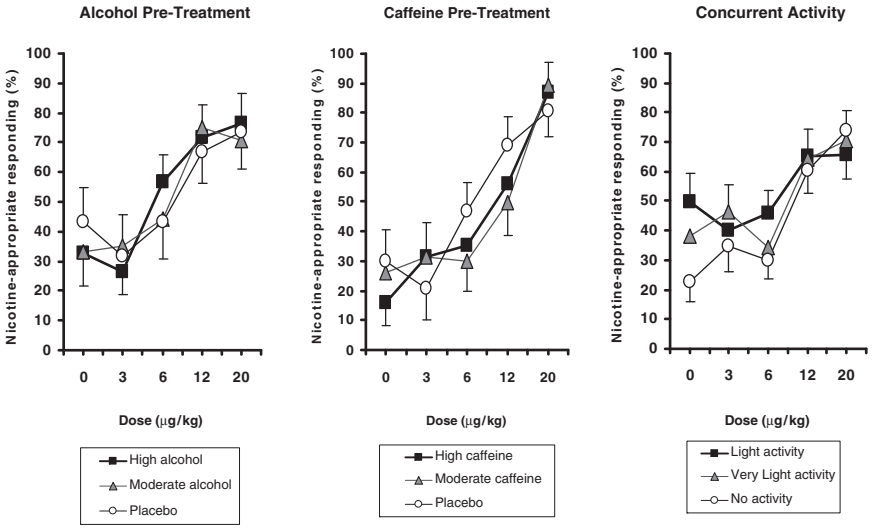

Fig. 5 *Left and middle*: Mean (±SEM) nicotine-appropriate responding across nicotine generalization doses (0–20 $\mu g\,kg^{-1}$) via nasal spray in the two-choice procedure as a function of alcohol (*left*, $n = 12$) or caffeine (*middle*, $n = 13$) pretreatment conditions. Reprinted with permission from Fig. 1 in Perkins et al. (2005a) published by Lippincott, Williams, and Wilkins, and from Fig. 1 in Perkins et al., (2005c) published by the American Psychological Association. *Right*: Mean (±SEM) nicotine-appropriate responding across nicotine generalization doses (0–20 $\mu g\,kg^{-1}$) via nasal spray in the two-choice procedure as a function of physical activity condition ($n = 17$). Reprinted with permission from Perkins et al. (2005b) of the Society for Research on Nicotine and Tobacco (see the journal's website: http://www.informaworld.com)

and 0.8 $g\,kg^{-1}$ alcohol, respectively, out of 24 total choices). Nicotine and alcohol each had main effects on several subjective responses, but virtually no interactions of nicotine by alcohol were observed, similar to the discrimination behavior. These results, which are consistent with recent rodent research (LeFoll and Goldberg 2005), suggest that the acute increase in smoking following alcohol consumption is not due to changes in nicotine's discriminative stimulus effects.

Caffeine pretreatment

We examined the influence of caffeine pretreatment on nicotine discrimination with virtually identical procedures: pretreating smokers with 0, 2.5, or 5.0 $mg\,kg^{-1}$ caffeine p.o. before each test of generalization, plus intermittent topping doses to maintain steady caffeine levels throughout generalization testing (Perkins et al. 2005c). As with the alcohol pretreatment study, caffeine pretreatment did not affect nicotine discrimination behavior in either the two-choice procedure, as shown in Fig. 5, or the three-choice procedure. Caffeine pretreatment also did not significantly affect nicotine choice (15.1, 13.0, and 13.7 choices following 0, 2.5, and 5.0 $mg\,kg^{-1}$ caffeine, respectively, out of 24 total choices). Subjective responses were influenced only by the main effects of nicotine and caffeine, not their interaction.

These findings are partly consistent with an earlier human study (Duka et al. 1998), in which pretreatment with a much smaller caffeine dose, 50 mg, versus placebo had no effect on discrimination of active nicotine generalization doses of 0.25, 0.5, or 1.0 mg via gum. That study also found only main effects of nicotine and caffeine on a few subjective effects. However, Duka et al. (1998) found that 50 mg caffeine pretreatment increased nicotine-appropriate responding after placebo gum, suggesting partial generalization between caffeine and nicotine. One animal study also found that caffeine enhanced nicotine-appropriate responding at a very low nicotine generalization dose but not at higher generalization doses, and caffeine did not generalize to nicotine (Gasior et al. 2002). In sum, concurrent caffeine intake appears to have little influence on nicotine discrimination.

3.2.3 Concurrent Physical Activity

Most testing during laboratory-based research on acute effects of abused drugs, including drug discrimination, is conducted when participants are in a resting state. Such a state provides a stable, quiet baseline for dependent measures from which to observe changes following acute drug administration. However, many drugs, including nicotine via tobacco smoking, are experienced by the user when physically active, such as walking, working, driving, etc. Our prior research had found that subjective arousal effects of nicotine, but not caffeine, reported by smokers at rest were not seen during mild physical activity (Perkins et al. 1994c). On the other hand, both physical activity and nicotine increase subjective arousal, and so interoceptive stimulus effects during activity could mimic some of those of nicotine. Therefore, effects of drug administration, including drug discrimination, when participants are at quiet rest may not generalize to drug effects when participants are engaged in physical activity. Yet, no human study had examined the influence of physical activity on drug discrimination responses.

We tested this idea with procedures very similar to the studies of pretreatment drug effects on nicotine discrimination (Perkins et al. 2005b). Smokers were trained to discriminate $20\,\mu g\,kg^{-1}$ from placebo and then responded to repeated tests of generalization of this discrimination across a range of nasal spray nicotine doses on subsequent days while concurrently engaged in different activity levels. These levels consisted of no activity (i.e., quiet rest), very light activity (15% of heart rate reserve), or light activity (30% of heart rate reserve, or the difference between resting heart rate and maximal heart rate). We tested subjects at these light levels of physical activity because few smokers experience the effects of nicotine while engaged in higher levels of activity (e.g., aerobic activity, which exceeds 50% of heart rate reserve). Physical activity involved pedaling a bicycle ergometer at a set speed and resistance. Subjects engaged in the nicotine choice procedure under these activity conditions after the last generalization trial, as in the prior studies of nicotine, alcohol, or caffeine pretreatment.

Results showed no significant effect of physical activity on nicotine discrimination responding in either the two-choice procedure, also shown in Fig. 5, or the

three-choice procedure. Physical activity tended to enhance nicotine-appropriate responding to placebo spray (Fig. 5), especially in women (not shown), suggesting some generalization of activity effects to nicotine, but this influence was not significant. Activity also did not alter nicotine choice (17.1, 16.0, and 15.5 choices following rest, very light, and light activity, respectively, out of 24 total choices.) Physical activity and nicotine had main effects, but no interactions, on subjective responses, as in the alcohol and caffeine pretreatment studies. Although the environmental context of physical activity had no influence on nicotine discrimination or self-administration, these findings contribute to the validity of laboratory-based research on nicotine and perhaps other drugs by showing that results of nicotine exposure observed in subjects at rest may in fact generalize well to the effects of nicotine in subjects engaged in light activity of the type common to daily tasks.

3.2.4 Other Potential Environmental Moderators

A more general way of viewing potential environmental moderators of nicotine discrimination is to consider them as distinct contexts for nicotine intake (see chapter by Stolerman, this volume). Thus, different training conditions or concurrent drug intake or activity levels may represent different contexts for discrimination of nicotine, as each can be seen as altering the exteroceptive stimulus context. However, concurrent drug use and physical activity also may alter the interoceptive context of discrimination by producing changes in interoceptive stimuli, such as through increases in arousal or fatigue. The fact that the contextual changes in our studies of alcohol, caffeine, or activity had little influence on nicotine discrimination does not diminish the potential importance of these or other contexts for discrimination. For example, in those studies, training and testing always took place in the same room and involved the same experimenter and method of drug administration, even if other elements of the context were systematically varied. More salient changes in the exteroceptive context could have more potent influences on discrimination behavior.

In perhaps the only other direct example in humans of an alteration in nicotine discrimination due to manipulation of an exteroceptive context, Duka et al. (2002) paired two different types of auditory stimuli ("elating" versus "depressing" music) with nicotine (1 mg) or placebo gum administration during acquisition of discrimination. Subjects were then tested for generalization in the presence of the nicotine- or placebo-paired music. A significant interaction of generalization dose by musical context was observed, as nicotine-appropriate responding was more strongly dose-dependent under the nicotine- versus placebo-paired music, although this was the case only when elating music was paired with nicotine and not when depressing music was paired with nicotine. Because the music did not actually alter mood, on the basis of subjective ratings, these results suggest that the auditory stimuli of the music served as an exteroceptive context for nicotine's effects, rather than producing an additional interoceptive context of mood. Similar moderation of drug discrimination by exteroceptive contexts has long been demonstrated in animal models (Jarbe et al. 1983).

Little other human research has directly examined the influence of other environmental factors that may moderate nicotine discrimination, although many such factors may exist. An obvious example is the other exteroceptive and interoceptive stimuli that commonly accompany nicotine intake via tobacco use. The sight, smell, and taste of cigarette smoke are salient stimuli that influence subjective responses to smoking, often to a greater extent than nicotine intake itself (Perkins et al. 2008; Rose 2006). Similarly, verbal information about the nicotine content of cigarettes can create a potent exteroceptive context that alters subjective effects related to nicotine discrimination (e.g., self-reported intensity of nicotine intake) more than the actual nicotine content of the cigarettes alters those effects (Perkins et al. 2008; see also Perkins et al. 2003). This issue is also important for methodological control in drug discrimination research, as isolating the interoceptive stimulus effects of a drug requires keeping constant the exteroceptive and other interoceptive stimuli involved in training and testing. Unintended exteroceptive or interoceptive stimuli (e.g., taste) accompanying a drug's interoceptive stimuli can introduce bias in discrimination test results (Abreu and Griffiths 1996).

4 Conclusions

Nicotine's interoceptive stimulus effects are clearly discriminable by humans and are centrally mediated. Although the threshold for this discrimination varies substantially between individuals – among nonsmokers as well as smokers – that threshold dose for most people is surprisingly low, well below the nicotine content of almost all commercial cigarette brands. Threshold doses for discrimination of nicotine via faster delivery methods, such as tobacco smoking, may be even lower. Factors that account for individual variability in nicotine discrimination have not received much attention but may include smoking status and sex, with nonsmokers and men possibly being more sensitive to the discriminative stimulus effects of moderate nicotine doses. Other potential individual difference characteristics related to nicotine discrimination may be genetics, obesity, and chronic use of other drugs.

Also underexplored is the influence of environmental factors that can acutely moderate nicotine discrimination. Training and testing conditions very strongly affect nicotine discrimination behavior, an observation that highlights the malleability of such behavior and serves to reinforce the need for strict control over experimental conditions during such research. Concurrent intake of other drugs may affect nicotine discrimination, although our research has shown little such influence. The fact that physical activity also does not alter nicotine discrimination suggests that findings on nicotine effects from studies of subjects at quiet rest, which comprise virtually all human research on drug discrimination, may generalize well to the effects of nicotine when subjects are engaged in light activity common to typical daily tasks.

Among correlates of nicotine discrimination, a few subjective effects, such as "head rush" or "buzzed", are often associated with discrimination behavior. However, such associations may differ depending on smoking status, sex, and other

factors. The relationship between nicotine discrimination and self-administration remains unclear, on the basis of the limited human research that has assessed both within the same study. Because nicotine discrimination behavior, or nicotine-appropriate responding, may be inversely, as well as directly, related to self-administration, it is important to bear in mind that the discriminative stimulus effects of nicotine do not necessarily reflect the hedonic valence of the drug (i.e., whether it is pleasurable or aversive).

These findings may be specific to the nasal spray procedure of nicotine administration, which has some strengths but also weaknesses as a tool for acute nicotine research. The data on the association of nicotine discrimination and self-administration presented here may be limited by the use of nicotine spray, which often failed to show clear reinforcement in smokers and is usually avoided by non-smokers. Future research needs to examine parameters of nicotine discrimination via other methods. In particular, nicotine intake via cigarette smoking certainly is more reinforcing than via nasal spray (Perkins et al. 1996b), and may have discriminative stimulus effects and associations with individual difference or contextual factors that are different from those of spray. Reasons for these differences could stem from the important sensory contributions of smoke inhalation (Rose 2006; Rose et al. 1999), as well as the difference in kinetics and route of administration (Henningfield and Keenan 1993). Other routes of administration and their accompanying sensory stimuli, such as smokeless tobacco, could lead to still other differences in the discriminative stimulus effects of nicotine intake from such products.

Moreover, an important, but completely unaddressed, question in this literature concerns whether discrimination of nicotine is required to see nicotine's reinforcement enhancing effect, or the increase in the reinforcing effects of unrelated stimuli in the presence of nicotine exposure (Chaudhri et al. 2006). This reinforcement-enhancing effect, potentially a major contribution to the persistence of smoking behavior, is separate from the primary reinforcing effects of nicotine as assessed via self-administration of a nicotine-containing substance. In other words, can a nicotine dose that is below the threshold for discrimination nevertheless result in enhancement in the reinforcing effects of another stimulus? Because this second type of reinforcing action of nicotine appears to be unrelated to its speed of administration, as in comparisons between bolus versus constant infusion (Chaudhri et al. 2006), discrimination of nicotine may not be necessary to observe the resulting reinforcement-enhancing effects of that nicotine.

Finally, the literature on human nicotine discrimination suggests that it may be important as an initial screening tool for medication development, such as by indicating a novel compound's site of action. However, the effect of pretreatment with a novel compound on nicotine discrimination per se will not necessarily predict the likely clinical efficacy of that compound, because of the uncertain association between discrimination and self-administration, among other unknowns in medication screening (see Perkins et al. 2006). This notion is supported by the fact that nicotine patch, an FDA-approved medication for smoking cessation, does not alter nicotine discrimination, while mecamylamine, a promising but as yet unapproved cessation medication, does block nicotine discrimination. Animal research

similarly shows that some medications or other treatments do (e.g., passive immunization, Malin et al. 2002), while others do not (e.g., bupropion, Young and Glennon 2002; rimonabant, LeFoll and Goldberg 2004), attenuate nicotine discrimination (see chapter by Stolerman, this volume). Such results further point to the likely complexity of the mechanisms of action of effective smoking cessation medications.

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Rodent Models of Nicotine Withdrawal Syndrome

David H. Malin and Pilar Goyarzu

Contents

Abstract Simple, rapid and inexpensive rodent models of nicotine physical dependence and withdrawal syndrome have proved useful for preliminary screening of smoking cessation treatments. They have led to an exponential increase of knowledge regarding the underlying neurobiological mechanisms of dependence and withdrawal syndrome. The human nicotine withdrawal syndrome in smoking cessation is variable and multidimensional, involving irritability, anxiety, depression, cognitive and attentional impairments, weight gain, sleep disturbances, and craving for nicotine. Aside from sleep disturbances, analogous phenomena have

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been seen in rodent models using different measures of withdrawal intensity. It appears likely that different withdrawal phenomena may involve some partially divergent mechanisms. For example, depression-like phenomena may involve alterations in mechanisms such as the mesolimbic dopamine pathway from the ventral tegmental area to the nucleus accumbens. Irritability and anxiety may involve alterations in endogenous opioid systems and other regions, such as the amygdala. This chapter reviews many additional anatomical, neurochemical, and developmental elements that impact nicotine physical dependence.

1 Introduction

Chronic use of nicotine, the major psychoactive ingredient in tobacco products, has been shown to cause dependence, and "nicotine dependence" is a recognized diagnostic category (American Psychiatric Association 1994) in the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV). "Nicotine withdrawal" is also a recognized disorder. It is only one of several criteria or components of nicotine dependence as defined in the DSM-IV (American Psychiatric Association 1994). This chapter will concentrate on laboratory studies of nicotine withdrawal syndrome, while other components of nicotine dependence, such as excessive self-administration of or craving for nicotine are covered in other chapters by Balfour and by LeFoll and Goldberg. The term "physiological dependence" as used in this chapter, is a state induced by chronic drug exposure that will result in a withdrawal syndrome upon discontinuation or reduction of the drug (Nutt et al. 1991). Unfortunately, this term can be quite misleading. The word "physiological" means that the tendency toward withdrawal syndrome is caused by a physiological adaptation to chronic drug exposure. It does not mean that all the signs and symptoms of withdrawal must be physiological in nature, such as a change in body temperature or blood pressure. The DSM-IV defines withdrawal as "a maladaptive behavioral change, with physiological and cognitive concomitants" (American Psychiatric Association 1994). Thus, physiological dependence may often be manifested by behavioral, cognitive, or emotional signs and symptoms following withdrawal from the drug.

For purposes of this chapter on animal research, a "withdrawal syndrome" is defined as a set of abnormal or impaired behaviors or physiological processes following the discontinuation of chronic drug exposure. The occurrence of such a withdrawal syndrome is the operational definition of physiological dependence. A human withdrawal syndrome resulting from smoking cessation has been described. Signs and symptoms include irritability, agitation, anxiety, sleep disturbances, difficulty concentrating, depression, weight gain, and craving for tobacco (Hughes 2007b). None of these states is specific to nicotine withdrawal alone; they can all occur in response to various other drugs or environmental stimuli. Therefore, it is not reasonable to expect that any rodent withdrawal sign be specific to nicotine withdrawal alone. The most striking aspect of the human withdrawal syndrome is

its multidimensional nature, with different individuals suffering from varying symptoms during smoking cessation. Therefore, we should not be surprised when different animal models of nicotine withdrawal syndrome measure different dimensions of emotional and behavioral alterations. It is a central thesis of this chapter that most of the same dimensions of nicotine withdrawal seen in smoking cessation have also been reflected in results from various rodent models. That is, almost all the states noted in the human syndrome have been detected in the animal laboratory through the corresponding measures or indicators of these states commonly employed in preclinical research. In this restricted sense, the models, viewed collectively, have achieved a certain construct validity, although they may lack face validity in terms of the contrasting means that produce nicotine dependence in laboratory rodents and human beings.

1.1 Relevance to Smoking Addiction and Cessation

There is little doubt that the nicotine's positive reinforcing actions, rather than physiological dependence, is the main factor in initiating a consistent tobacco habit (see chapters by Balfour and by LeFoll and Goldberg in this volume). Physiological dependence commonly manifests itself later as a withdrawal syndrome when tobacco products are unavailable or when smoking cessation is attempted. In fact, smoking cessation produces emotional distress comparable to that typically experienced by psychiatric outpatients (Hughes 2006). The withdrawal syndrome complicates the smoking cessation process and creates an additional incentive for relapse to tobacco use. Should the patient lapse, even briefly, renewed tobacco use will result in an unusually strong reinforcing effect, since positive reinforcement will be supplemented by "negative reinforcement": reinforcement resulting from the removal of an aversive condition (the withdrawal syndrome). In unaided quit attempts, relapse is extremely common during the first week of cessation, during the usual time-course of the withdrawal syndrome (Hughes et al. 2004). Also, in many, but not all, studies, the severity of withdrawal symptoms were predictive of relapse to smoking (Hughes 2007b; Piasecki et al. 2000, 2003). Therefore, management of physical dependence and withdrawal syndrome is one important element in designing comprehensive plans and procedures for smoking cessation.

1.2 Purpose of Animal Models

Animal models of physiological dependence induction and withdrawal syndrome can be used for preliminary screening of potential therapeutic measures to support smoking cessation. In addition, the development of rational therapies depends on knowledge of biological mechanisms underlying physical dependence and withdrawal syndrome. Animal models can be used to test various hypotheses regarding such underlying mechanisms.

1.3 Scope of This Review

This review will discuss many, but not all, of rat or mouse models of physical dependence on and withdrawal from chronic nicotine treatment. It will discuss the various means of testing and evaluating physical dependence, as well as the knowledge gained from the increasing use of such models. The main emphasis will be on studies published since an earlier review article (Malin 2001). The use of these models has increased so much since that time that an exhaustive and complete review of such research is no longer possible. The authors apologize for any omissions. In particular, we have omitted many studies that reported biochemical alterations after termination of nicotine exposure, without ascertaining that the regimen of nicotine exposure had resulted in a behavioral withdrawal syndrome. In studying the effect of nicotine exposure on various biomarkers, it is desirable to include behavioral withdrawal measures; this ensures that the parameters of nicotine exposure are relevant to physical dependence. Alternatively, one can employ nicotine exposure parameters that have previously been demonstrated to result in a withdrawal syndrome.

2 Types of Rodent Models

Since operational definition of physical dependence is the tendency to display a withdrawal syndrome after drug termination, the severity of the withdrawal syndrome is the most commonly used indicator of the degree of physical dependence. Rodent models differ in at least three dimensions: the means used to induce physical dependence, the means subsequently used to induce a withdrawal syndrome and the variables used to estimate the severity of the withdrawal syndrome. Two models may be identical in one of these respects, yet totally different in another.

2.1 Means of Inducing Dependence and Abstinence

By far most commonly employed means of inducing nicotine dependence in the rat is continuous subcutaneous infusion via osmotic minipump (Malin et al. 1992). Infusion rates typically range from 3 to 9 $mg\,kg^{-1}\ day^{-1}$ of nicotine bitartrate (from 1.05 to 3.15 $mg\,kg^{-1}\ day^{-1}$ expressed as the nicotine base). The higher rate has been more reliable for producing clear-cut dependence in the authors' laboratory. This procedure releases nicotine at a constant rate for one or more weeks. In validating such models, it was essential to provide a control condition where rats receive the same minipumps implanted by the same procedure, but filled with solvent alone (generally isotonic saline). This method might seem to lack external validity, since tobacco users self-administer nicotine-containing products in discrete episodes. However, it has been shown that smokers "titrate" their tobacco consumption so as to maintain rather steady blood levels of nicotine (Benowitz 1990).

The blood concentrations resulting from a standard infusion rate of 9 mg kg^{-1} day^{-1} result are almost identical concentrations to those that have been measured in heavy smokers, smoking at their *ad lib* rate (Benowitz et al. 1982; LeSage et al. 2002). Of course, one difference is that, in the smokers' case, the levels decline during daily sleep. On the other hand, it is difficult to argue that a week of continuous nicotine exposure results in an unrealistically high amount of total nicotine exposure, as compared to years of 16 or 17 h per day of continuously elevated nicotine blood concentrations in human heavy smokers.

The continuous release method has several advantages. It has resulted in a wide variety of withdrawal signs that relate to several dimensions of human withdrawal phenomena. This includes a large majority of the withdrawal severity measures described in Sect. 2.3. It produces readily detectable dependence quickly and conveniently, in a week or less, without any daily injections or other interventions that might repeatedly stress the subject. The complete lack of discrete stimuli during the period of drug exposure helps to rule out learning or conditioning theory interpretations of dependence formation. This allows more focus on physical/chemical changes in the central nervous system as explanations for acquisition of dependence.

On the other hand, discrete episodes of nicotine administration have also resulted in subsequent withdrawal phenomena. For example, reduced social interaction has been induced by termination of a 14-day series of b.i.d. i.p. injections (Costall et al. 1990a). Termination of a 10-day series of b.i.d. s.c. injections resulted in an anxiogenic response (Pandey et al. 2001). There was also a significant reduction of locomotor activity a day following termination of nicotine-containing liquid diets (Halladay et al. 1999). Other studies have attempted to determine whether voluntary nicotine consumption in the rat can result in physical dependence and withdrawal syndrome. Somatically expressed behavioral abstinence signs, both spontaneous, and mecamylamine-precipitated were observed following prolonged operant intravenous self-administration (IVSA) of nicotine under daily limited access conditions (Paterson and Markou 2004). Precipitated abstinence signs were also observed following IVSA with 23 h day^{-1} access (O'Dell and Koob 2007).

Nicotine dependence models employing mice are increasingly desirable because of the availability of genetically engineered mouse strains. Continuous subcutaneous infusion, via smaller osmotic minipumps, has resulted in subsequent withdrawal signs in mice (Balerio et al. 2004; Damaj et al. 2003; Davis et al. 2005; Kota et al. 2007; Semenova et al. 2003). Physical dependence in mice has also been induced by chronic or subchronic injection series (Biala and Weglinska 2005; Costall et al. 1990a; Isola et al. 1999). Chronic consumption of nicotine in drinking water likewise resulted in subsequent withdrawal signs in mice (Fornari et al. 2007; Grabus et al. 2005; Halladay et al. 1999; Salmon et al. 2004).

2.2 Means of Inducing Nicotine Withdrawal

Both spontaneous and nicotinic antagonist-precipitated withdrawal have been widely used in both rats and mice. Spontaneous withdrawal is initiated by simply

terminating nicotine administration. In a majority of cases withdrawal signs of various types in both rats and mice have been reported primarily between 1 and 3 days after initiation of withdrawal, with some reports as early as 12 h or as late as 4 days (Catania et al. 2003; Cheeta et al. 2001; Damaj et al. 2003; Grabus et al. 2005; Halladay et al. 1999; Isola et al. 1999; Malin et al. 1992; Skjei and Markou 2003). One notable exception is a report of withdrawal signs persisting for several weeks after certain termination of certain regimens of intravenous nicotine self-administration (Paterson and Markou 2004).

Nicotine abstinence signs may also be precipitated within minutes by administration of nicotinic receptor antagonists. This has been accomplished most often with the noncompetitive antagonist mecamylamine (Hildebrand et al. 1999; Lake et al. 2002; Malin et al. 1994; Suzuki et al. 1996). Doses around $1\,mg\,kg^{-1}$ s.c are sufficient to precipitate withdrawal signs in nicotine-infused rats, while a dose of $6\,mg\,kg^{-1}$ s.c. caused similar behavioral signs (a quasi-nicotine abstinence syndrome) in nicotine-naive rats (Malin et al. 1994). However, nicotine abstinence signs have also been precipitated by competitive nicotinic receptor antagonists such as dihydro-beta-erythroidine (DHβE), methyllycaconitine (MLA) and intraventricular hexamethonium (Damaj et al. 2003; Malin et al. 1997, 1998a), and even by opiate receptor antagonists (Malin et al. 1993a). The implications of these studies for the role of various receptor subtypes will be discussed later in this chapter.

2.3 Measures of Nicotine Withdrawal Syndrome Severity

The majority of withdrawal signs and symptoms in human smokers fall into categories of irritability/agitation, anxiety, depression, cognitive/attentional disturbances, weight and appetite gain, and sleep disturbances (Hughes 2007b). The various models of nicotine dependence and withdrawal syndrome have resulted in marked effects on rodent measures of all the above dimensions except sleep disturbance, which does not seem to have been studied in the published literature.

Irritability refers to exaggerated responses to internal or external stimuli that would ordinarily be better tolerated. Irritability and agitation are among the most commonly reported symptoms during smoking cessation (Hughes 2007a). Spontaneously emitted behaviors (Malin et al. 1992) such as writhes, gasps, shakes, tremors, teeth chattering, vacuous chewing, ptosis, and scratching are among the most frequently reported abstinence signs in rat models of nicotine withdrawal (see Table 1). It has been suggested that such signs are "somatic" as opposed to "affective" in nature and merely reflect peripheral bodily changes in withdrawal rather than a centrally-mediated emotional state (Epping-Jordan et al. 1998; Watkins et al. 2000). On the other hand, there are several reasons to suggest that these "somatic" signs may reflect a centrally-mediated dysphoric state of heightened irritability:

1. The fact that an animal's abnormal behavior is expressed through a bodily movement pattern does not automatically mean that there is a peripheral disorder of

Table 1 Dependence induction methods

Spontaneous or precipitated	Withdrawal syndrome measures	Some representative studies
Continuous subcutaneous infusion in rats		
Spontaneous	Somatically expressed behaviors	Besheer and Bevins (2003); Epping-Jordan et al. (1998); Harrison et al. (2001); Malin et al. (1992)
	Self-stimulation (ICSS) threshold	Bruijnzeel et al. (2007); Cryan et al. (2003); Epping-Jordan et al. (1998); Harrison et al. (2001); Skjei and Markou (2003)
	Reduced novelty reward (1h int)	Besheer and Bevins (2003)
	Social interaction (reduced)	Cheeta et al. (2001)
	Increased body weight	Grunberg et al. (1986); Harrison et al. (2001); Malin et al. (1992)
	Increased food consumption (females)	Grunberg et al. (1986)
	Reduced progressive ratio breakpoint	LeSage et al. (2006)
	Increased auditory startle response	Helton et al. (1993); Rasmussen et al. (2000);
	Sustained attention (5-CSRTT)	Shoaib and Bizarro (2005)
Precipitated mecamylamine (mec)	Somatically expressed behaviors	Besheer and Bevins (2003); Hildebrand et al. (1998); Malin et al. (1994); Suzuki et al. (1996)
	Conditioned place aversion	Göktalay et al.(2006); Suzuki (1996)
	Disrupted operant behavior F-R 10	Vann et al. (2006)
	ICSS threshold	Bruijnzeel et al. (2007)
	Open field thigmotaxis	Tzavara et al. (2002)
	Conditioned ICSS threshold	Kenny and Markou (2005)
Precipitated mec and chlorisondamine	Somatically expressed behaviors	Watkins et al. (2000)
	Self-stimulation (ICSS) threshold	Watkins et al. (2000)
Precipitated intra accumbens mec	Hyperalgesia	Schmidt et al. (2001)
Precipitated DHβE in VTA	Self-stimulation (ICSS) threshold	Brujijnzeel and Markou (2004)
Precipitated DHβE	Conditioned ICSS threshold	Kenny and Markou (2005)
	Sustained attention (5-CSRTT)	Shoaib and Bizarro (2005)
Precipitated mec, DHβE and naloxone	Conditioned place aversion	Watkins et al. (2000)
Continuous subcutaneous infusion in mice		
Spontaneous	Somatically expressed behaviors	Damaj et al. (2003); Kota et al. (2007)
	Anxiety	Damaj et al. (2003); Kota et al. (2007)
	Hyperalgesia	Damaj et al. (2003); Kota et al. (2007)
	Contextual fear conditioning	Davis and Gould (2007)
	Increased jumping	Semenova et al. (2003)
	Decreased prepulse inhibition	Semenova et al. (2003)

(continued)

Table 1 (continued)

Spontaneous or precipitated	Withdrawal syndrome measures	Some representative studies
Precipitated	Somatically expressed behaviors	Balerio et al. 2004; Berrendero et al. (2005); Castañé et al. (2002); Damaj et al. (2003); Kota et al. (2007)
	Conditioned place aversion	Balerio et al. 2004
	Anxiety	Damaj et al. (2003); Kota et al. (2007)
	Hyperalgesia	Damaj et al. (2003); Kota et al. (2007)
	Contextual fear conditioning	Davis et al. (2005)
Injection series in rats		
Spontaneous	Reduced social interaction	Costall et al. (1990a); Irvine et al. (1999)
	Elevated plus maze (anxiety)	Pandey et al. (2001)
	Generalization to PTZ (anxiety)	Harris et al. (1986)
Injection series in mice		
Spontaneous	Somatically expressed behaviors	Isola et al. (1999); Mannucci et al. (2005, 2006)
	Decreased activity	Isola et al. (1999); Manucci et al. (2006)
	Increased food consumption	Mannucci et al. (2005)
	Light/dark exploration (anxiety)	Costall et al. (1990a)
	Forced swimming (depression)	Manucci et al. (2006)
Precipitated	Somatically expressed behaviors	Biala et al. (2005); Isola et al. (1999)
	Increased weight	Biala et al. (2005)
	Decreased activity	Biala et al. (2005)
	Anxiety	Biala et al. (2005)
Drinking water in mice		
Spontaneous	Somatically expressed behaviors	Grabus et al. (2005)
	Hyperalgesia	Grabus et al. (2005)
	Increased weight	Fornari et al. (2007)
Precipitated	Somatically expressed behaviors	Grabus et al. (2005); Salmon et al. (2004)
Liquid diet in rats		
Spontaneous	Decreased activity	Halladay et al. (1999)
Operant I.V. self-administration in rats		
Spontaneous	Somatically expressed behaviors	Paterson and Markou (2004)
	Increased self-administration	O'Dell and Koob (2007)
Precipitated	Somatically expressed behaviors	O'Dell et al. (2007); O'Dell and Koob (2007)
Nicotine pellet implant in rats		
Spontaneous	Increased body weight (female)	Levin et al. (1987)
	Increased food consumption (female)	Levin et al. (1987)

the body. For example, when a rat in opiate withdrawal repeatedly scratches itself, this does not indicate a disorder of the paw. Rather it indicates heightened nervous system sensitivity to otherwise innocuous cutaneous stimuli. Likewise, when a person gasps with surprise, trembles with fear, or pounds the table in anger, the bodily motions reflect a centrally mediated emotional/motivational state.

2. As described later in this section, the exact parameters of nicotine exposure that induce these signs have been shown to be aversive and to produce exaggerated responses to mildly aversive stimuli.
3. Injection of the opiate antagonist naloxone precipitates the "somatic" signs of nicotine withdrawal (Adams and Cicero 1998; Carboni et al. 2000; Malin et al. 1993a), but not "affective" signs, such as intracranial self-stimulation (ICSS) threshold elevation (Watkins et al. 2000). Yet naloxone injection in a nicotine-infused rat is robustly aversive (Ise et al. 2000; Watkins et al. 2000). Therefore, the "somatic" signs may actually correspond better than ICSS thresholds to an aversive motivational state.
4. Naloxone precipitation, unlike nicotine-antagonist challenge, fails to decrease DA-activity, yet still precipitates "somatic" signs (Carboni et al. 2000). Thus the aversiveness of nicotine withdrawal can be dissociated in part from those effects on the mesolimbic-DA reinforcement pathway that have sometimes been proposed to account for the "affective" aspects of withdrawal (Balfour 2004; Paterson et al. 2007).
5. As described in Sect. 4, the "somatic" signs appear to depend a major extent on central nervous system mechanisms.
6. It is well known that irritability and dysphoria are major emotional features of opiate narcotic withdrawal. Almost all of the somatically expressed behaviors seen in rat nicotine withdrawal are commonly observed in rat opiate withdrawal (Aricioglu-Kartal et al. 2003; Malin et al. 1990; Xiang et al. 2006). Yet the bodily physiology in these two syndromes is quite different and even opposite. For example blood pressure rises in narcotic withdrawal and falls in nicotine withdrawal. Many prominent peripheral physiological changes in opiate withdrawal result from overstimulation of the sympathetic nervous system (Dellu and Thorén 1987). This does not happen in nicotine withdrawal, since nicotinic acetylcholine receptors and opiate receptors play opposite roles in regulating the peripheral sympathetic nervous system. Thus, peripheral physiology appears to be largely dissociable from the "somatic" behavioral signs.

While we realize that some others will disagree, we suggest that it cannot be automatically assumed that the "somatic" signs cannot reflect a centrally-mediated emotional or motivational state. For this reason, we refer to them not as "somatic signs," but as "somatically expressed behaviors."

Another, particularly direct, indication of irritability is the heightened startle response observed following the termination of nicotine infusion (Helton et al. 1993; Rasmussen et al. 2000). In addition, hyperalgesia, heightened responsiveness to painful or irritating stimuli, has been reported in several rodent studies of nicotine abstinence (Damaj et al. 2003; Grabus et al. 2005; Kota et al. 2007; Schmidt et al. 2001).

Several standard measures of anxiety have been evaluated in rodent nicotine abstinence models. This includes avoidance of open arms on the elevated plus maze (Biala and Weglinska 2005; Damaj et al. 2003; Pandey et al. 2001), avoidance of the light side in a light/dark apparatus (Costall et al. 1990a), reduced social interaction (Cheeta et al. 2001; Costall et al. 1990a; Irvine et al. 1999), thigmotaxis, avoidance of central areas in an open field, (Tzavara et al. 2002), and generalization to the anxiety-inducing compound pentylenetetrazole (PTZ) in the drug discrimination paradigm (Harris et al. 1986). Escape jumping seen in a mouse nicotine model may also reflect extreme anxiety and/or irritability (Semenova et al. 2003).

A number of nicotine abstinence models have produced behavioral changes highly suggestive of a depression-like state. This includes depressed locomotor activity (Biala and Weglinska 2005; Halladay et al. 1999; Isola et al. 1999; Malin et al. 1992; Mannucci et al. 2006), decreased coping activity in the forced swim test, which is often used to screen potential antidepressant medications (Mannucci et al. 2006), reduced novelty reward effect (Besheer and Bevins 2003), reduced progressive ratio breakpoint for food reward, indicative of reduced sensitivity to reinforcement (LeSage et al. 2006), and increased intracranial self-stimulation (ICSS) thresholds, a model of anhedonia, one common component of depression (Bruijnzeel and Markou 2004; Bruijnzeel et al. 2007; Cryan et al. 2003; Epping-Jordan et al. 1998; Harrison et al. 2001; Skjei and Markou 2003; Watkins et al. 2000). Even exposure to environments associated with nicotine withdrawal can raise ICSS thresholds (Kenny and Markou 2005).

Attentional or cognitive impairments have also been observed in rodent models of nicotine withdrawal. These include impaired performance of a test of sustained attention (Shoaib and Bizarro 2005), disrupted contextual fear conditioning (Davis and Gould 2007; Davis et al. 2005), disrupted operant behaviors (Vann et al. 2006), and decreased prepulse inhibition, a test of selective attention (Semenova et al. 2003).

As mentioned above, weight gain and increased appetite are often experienced during smoking cessation. Rodent models of nicotine withdrawal have also resulted in increased food consumption (Grunberg et al. 1986; Levin et al. 1987; Mannucci et al. 2005) and in weight gains (Biala and Weglinska 2005; Fornari et al. 2007; Grunberg et al. 1986; Harrison et al. 2001; Levin et al. 1987; Malin et al. 1992).

The question remains, are the nicotine withdrawal phenomena in the rodent models sufficiently aversive to exert a motivational effect? This can be tested by whether rodents will avoid an environment associated with precipitated nicotine abstinence syndrome, in comparison with an environment associated only with a saline injection. Significant place aversion conditioned to nicotine withdrawal has been repeatedly observed in rodent models (Balfour 2002; Göktalay et al. 2006; Malin et al. 2006; Suzuki et al. 1996; Watkins et al. 2000).

Another important question is the relationship between nicotine abstinence and nicotine self-administration. O'Dell and Koob (2007) provided 23 h day^{-1} access to intravenous nicotine self-administration for 4-day intervals with intervening 3-day intervals of nicotine abstinence. This resulted in somatically expressed behavioral signs as well as heightened nicotine self-administration on the first day following

enforced abstinence. This may possibly model a motivational effect of nicotine abstinence that contributes to nicotine craving and relapse to tobacco use.

3 Issues of Validity

To what extent are the rodent models of nicotine physical dependence and nicotine withdrawal syndrome valid representations of physical dependence and withdrawal syndrome in human tobacco users? Validity can only be partial at best because of species differences and differences in the means, duration and composition of drug exposure (nicotine alone versus tobacco smoke). The differences between the models and human tobacco use phenomena will always be as important as the analogies.

In addressing this issue, we must differentiate several types of validity, such as face, internal and external validity. At first glance, most rodent models would seem to lack face validity, since different methods are used to induce dependence and since there are few topographically similar behaviors seen in human tobacco cessation and rodent nicotine abstinence. This is to be expected, however, since rodents and human beings tend to express similar emotional states through differing behaviors. For example, rodent anxiety is often measured by thigmotaxis (staying close to boundary walls), which is hardly a common anxiety measure in human beings. However, one can view the human tobacco cessation syndrome not only as a set of specific behaviors, but as a set of changes in basic psychological states (irritability/agitation, anxiety, depression, cognitive/attentional disturbances, and appetite). These states are usually measured by different means in human beings and rodents. Therefore, one category of validity issue is defined by the question: to what extent is the same constellation of psychological states present in rodent withdrawal models and human nicotine withdrawal during smoking cessation, even if indicated by different measures? As noted in the previous section, standard rodent indicators for all of the above states have been altered in the same direction as in smoking cessation in one or another version of the rodent models of withdrawal syndrome. In particular, almost all of these changes have been noted in models that induce dependence by continuous s.c. nicotine infusion and induce withdrawal by termination of infusion or by injection of nicotinic receptor antagonist (Damaj et al. 2003; Epping-Jordan et al. 1998; Malin et al. 1992, 1994). From this perspective, the collective variations of this basic rodent model (measuring different dimensions of the withdrawal syndrome) demonstrate a degree of construct validity.

Internal validity of the rodent models involves verifying that withdrawal severity (indicated by numbers of observed behavioral changes) reflects chronic nicotine exposure followed by termination of that exposure. The rat model involving continuous nicotine infusion has probably been the most extensively validated in this sense (Malin 2001), meeting the following validity criteria:

1. There are significantly more signs on the day or days immediately following termination of drug infusion than before infusion, during infusion, or following a subsequent recovery period (Malin et al. 1992).

2. There are more abstinence signs following nicotine infusion than equivalent saline infusion, as well as more abstinence signs after higher rates of infusion (Malin et al. 1992).
3. Nicotine abstinence can be potently and promptly reversed by injection of nicotine (Malin et al. 1992).
4. The dependence induced by nicotine infusion can be prevented by coinfusion with the nicotinic receptor antagonist mecamylamine (Malin 2001).
5. An abstinence syndrome can be promptly precipitated in nicotine-infused rats by blocking nicotinic receptors with the competitive antagonist DHβE (Epping-Jordan et al. 1998; Malin et al. 1998a), or by inactivating them with noncompetitive antagonists mecamylamine (Malin et al. 1994), hexamethonium (Malin et al. 1997), or chlorisondamine (Hildebrand et al. 1997).

All of these results are consistent with the hypothesis that the withdrawal severity (numbers of withdrawal signs) reflects chronic overstimulation of nicotinic cholinergic receptors followed by reduced stimulation.

Finally, there is the difficult question of "external validity": the degree of relevance to dependence and withdrawal syndrome in actual human smokers. External validity can only be established incrementally by noting phenomena demonstrated in a given model that are also seen in physically dependent human tobacco users and vice versa. For example, the rat continuous infusion model has predicted one effect (Malin et al. 1993a) that was subsequently observed in human smokers: the precipitation of an abstinence syndrome by injection of the opiate receptor antagonist naloxone in smokers, but not in nonsmokers (Krishnan-Sarin et al. 1999) Also, an extensive study was carried out specifically to test the external validity of the rat continuous infusion model by determining whether bupropion would have effects in that model analogous to its effects in human smokers (Malin et al. 2006). Bupropion reduces the withdrawal syndrome during smoking cessation (Shiffman et al. 2000). Since bupropion treatment is usually initiated prior to the target quit date, rats that were already nicotine-dependent were coinfused with bupropion for 7 days prior to mecamylamine challenge. This largely prevented the mecamylamine-precipitated abstinence syndrome. The aversiveness of that syndrome was also virtually eliminated based on conditioned place aversion to a compartment associated with the mecamylamine treatment. This lack of response to the nicotinic antagonist suggests that bupropion had attenuated the state of nicotine dependence. In a third experiment, an ongoing spontaneous nicotine abstinence syndrome was largely reversed by bupropion (Malin et al. 2006). Cryan et al. (2003) also reported that bupropion attenuated the raised ICSS thresholds as well as the somatically expressed behavioral signs observed during nicotine abstinence. Because of species differences, as well as differences in the means and duration of nicotine exposure, external validity of the animal models can be partial at best. However, any interesting phenomenon seen in one or more animal models may well be worth assessing in studies with human subjects.

4 Anatomical Correlates

The availability of rodent models of nicotine physical dependence and abstinence has made it possible to identify several anatomical regions that play a critical role in these phenomena. In reviewing the evidence, the multidimensional nature of the nicotine withdrawal syndrome should be considered, raising the possibility that different withdrawal signs and symptoms might be attributable to events in different anatomical regions. Table 2 summarizes a number of representative studies. There is some overlap with Table 3 on neurochemical mechanisms, since many studies cited there are studies of regional neurochemistry.

The nicotinic antagonist hexamethonium, which does not readily cross the blood–brain barrier, precipitated a nicotine abstinence syndrome with extraordinary potency through third ventricle administration in nicotine-infused, but not saline-infused rats. By peripheral administration, it had no selective effect on nicotine-infused rats. This strongly suggests a central site of action. On the other hand, peripherally administered chlorisondamine, which also does not readily cross the blood–brain barrier, has been shown to precipitate somatic nicotine abstinence signs selectively in nicotine-infused rats (Hildebrand et al. 1997). Watkins et al. (2000) also precipitated somatically expressed behavioral abstinence signs, but not ICSS threshold elevations by i.c.v. (lateral ventricle) infusion of chlorisondamine. Nevertheless, it required a far higher dose to precipitate a somatically expressed abstinence syndrome by the peripheral route than by central administration. This would not be the case if the site of action were only peripheral. The evidence clearly suggests a major central nervous system component in nicotine physical dependence and abstinence syndrome, while the existence of an additional peripheral component remains a distinct possibility.

Within the brain, the mesolimbic dopamine (DA) pathway has been the most frequent target of investigation, since it is potently stimulated by nicotine and since it is believed to be critically important in nicotine positive reinforcement (Di Chiara 2000). This pathway originates in the ventral tegmental area (VTA) and has its primary terminus in the nucleus accumbens (NAcc). There is ample evidence of changes in the NAcc during rodent nicotine withdrawal. In contrast to the immediate effect of nicotine, nicotine withdrawal results in decreased dopamine output in the NAcc (Carboni et al. 2000; Hildebrand et al. 1998; Rahman et al. 2004). This would appear to be consistent with the increased ICSS thresholds in this same pathway (Epping-Jordan et al. 1998). In addition, during nicotine withdrawal in the rat, there are highly localized increases in NAcc blood flow, as indicated by the fMRI BOLD technique (Shoaib et al. 2004). Also during withdrawal, the NAcc becomes insensitive to nicotine stimulation, as indicated by its cFos response (Salminen et al. 1999). Injection of mecamylamine directly into the NAcc precipitates hyperalgesia in nicotine-dependent rats (Schmidt et al. 2001) However, aside, from this effect on pain sensitivity, these changes in the NAcc might be “downstream” consequences of altered input from the VTA.

Mecamylamine injected into the VTA of nicotine-dependent rats induced decreased DA output in the NAcc (Hildebrand et al. 1999), while injection directly

Table 2 Anatomical regions involved in nicotine withdrawal

Anatomical region	Withdrawal measure or neurochemical correlate	Some representative references
Nucleus Accumbens	Reduced extracellular DA	Carboni et al. (2000); Rahman et al. (2004)
	Hyperalgesia precipitated by NAcc mec + B36	Schmidt et al. (2001)
	fMRI BOLD (blood oxygen)	Shoaib et al. (2004)
	Fos response becomes insensitive to nicotine	Salminen et al. (1999)
	Reduced DA output in NAcc	Hilderbrand et al. (1998)
Prefrontal cortex	Increased DA output	Carboni et al. (2000)
Striatum	Fos response becomes insensitive to nicotine	Salminen et al. (1999)
Ventral Tegmental Area	ICSS thresholds increased by DHβE in VTA	Bruijnzeel and Markou (2004)
	ICSS threshold response to D1 antagonist	Bruijnzeel and Markou (2005)
	Somatically expressed behaviors (precipitated by mec in VTA)	Hildebrand et al. (1999)
	Reduced DA output in NAcc (precipitated by mec in VTA)	Hildebrand et al. (1999)
	Reduced locomtor activity	Hildebrand et al. (1999)
	Reduced locomotor activity by methyllycaconitine in VTA	Nomikos et al. (1999)
Amygdala	c-fos induction during mec-precipitated withdrawal	Panagis et al. (2000)
	Reduced extracellular DA during mec-precipitated withdrawal	Panagis et al. (2000)
	Light avoidance (anxiety) reduced by ondansetron to amygdala	Costall et al. (1990a)
Dorsal Raphe' Nuc.	Social interaction (restored by nicotine in DRN)	Cheeta et al. (2001)
	Light avoidance (anxiety) reduced by ondansetron to DRN	Costall et al. (1990a)
Cingulate Gyrus	Fos response becomes insensitive to nicotine	Salminen et al. (1999)
Peripheral	Somatically expressed behaviors precipitated by chlorosondamine	Watkins et al. (2000)

into the NAcc failed to do so. In addition, the injection of several different nicotinic receptor antagonists into the VTA of nicotine-dependent rodents, resulted in somatically-expressed withdrawal behaviors (Hildebrand et al. 1999), reduced locomotor activity (Hildebrand et al. 1999; Nomikos et al. 1999), and increased ICSS thresholds (Bruijnzeel and Markou 2004). The electrical activity of VTA dopaminergic neuron decreased during the first day of withdrawal in the rat following a nicotine injection series (Liu and Jin 2004). This might help account for the decrease in NAcc dopamine release described below. However, Rasmussen and Czachura (1995) reported that the firing rate of VTA neurons, though depressed during continuous nicotine infusion, recovered to baseline levels during withdrawal,

Table 3 Neurochemical mechanisms involved in nicotine withdrawal

Neurochemical mechanism	Withdrawal measure	Some representative references
ACh and AChR		
Upregulation of general nicotinic receptors (H3-nic binding)	Termination of 4 or 7 weeks nicotine in drinking water	Pietilä et al. (1998)
Increased ACh release in NAcc	Mec-precipitated somatically expressed withdrawal behaviors	Rada et al. (2001)
α4β2 nAChR	ICSS threshold	Epping-Jordan et al. (1998)
	Mec-precipitated somatically expressed behaviors	Cohen et al. (2003)
	DHβE in VTA precipitated increased ICSS threshold	Bruijnzeel and Markou (2004)
	DHβE i.cv. precipitated somatically expressed behaviors	Malin et al. (1998b)
β2 nAChR	β2 gene deletion does not affect somatically expressed behaviors	Besson et al. (2006); Salas et al. (2004)
β4 nAChR	β4 gene deletion eliminates somatically expressed behaviors	Salas et al. (2004)
α7 nAChR	Increased α7 agonist hippocampal NE release	Barik and Wonnacott (2005)
	MLA does not precipitate withdrawal, somatic expressed behaviors and ICSS thresholds	Markou and Paterson (2001)
	Reduced locomotor activity by methyllycaconitine (MLA) in VTA	Nomikos et al. (1999)
Serotonin		
5HT turnover (5HIAA/5HT) reduced	Spontaneous withdrawal in mouse	Yasuda et al. (2002)
5HTP precursor, 5HTP (effect preventable by 5HT1A antagonist)	Reversed withdrawal-induced immobility in forced swim test	Mannucci et al. (2006)
Nicotine in dorsal raphe'	Reversed withdrawal-induced anxiety (social interaction test)	Cheeta et al. (2001)
5HT3 antagonist in amygdala and dorsal raphe'	Reduced withdrawal-induced anxiety (light avoidance)	Costall et al. (1990a)
5-HT3 antagonist	Reduced withdrawal-induced conditioned place aversion	Suzuki et al. (1997)
Reduced diencephalic 5HT1A receptors	50 and 30 days after nicotine withdrawal in mice	Mannucci et al. (2006)
Fluoxetine and 5HT1A antagonist	Reversed withdrawal-induced increased ICSS thresholds	Harrison et al. (2001)
5HT1A antagonist	Reversed withdrawal-induced increased in auditory startle	Rasmussen et al. (2000)
Increased sensitivity to 5HT2 agonist	Wet-dog shakes	Suemaru et al. (2001)
	Head-twitches in spontaneous and precipitated, withdrawal (mec and DHβE)	Yasuda et al. (2002)

(continued)

Table 3 (continued)

Catecholamine transmitters		
Decreased DA release in NAcc	Mec-precipitated somatically expressed withdrawal behaviors	Rada et al. (2001)
Decreased NAcc DA output	Mec-precipitated somatically expressed withdrawal behaviors	Carboni et al. (2000)
Increased medial prefrontal cortex DA output	Mec-precipitated somatically expressed withdrawal behaviors	Carboni et al. (2000)
No change NAcc DA output	Naloxone-precipitated somatically expressed withdrawal behaviors	Carboni et al. (2000)
Reduced NAcc DA output	Somatically expressed behaviors precipitated by mec in VTA	Hilderbrand et al. (1999)
Decreased sensitivity to D1-like receptor antagonist	ICSS threshold changes induced by DA antagonist SCH3390-	Bruijnzeel and Markou (2005)
Reduced NAcc DA output (prevented by nic. antibody)	Withdrawal precipitated by systemic mec	Lindblom et al. (2005)
Decreased firing rate of VTA DA neurons	Spontaneous withdrawal	Liu and Jin (2004)
Firing rate of substantia nigra DA neurons increased	Spontaneous withdrawal	Rasmussen and Czachura (1995)
Recovery from depressed firing rate of VTA DA neurons	Spontaneous withdrawal	Rasmussen and Czachura (1995)
D1-like antagonist in amygdala doesn't precipitate withdrawal	ICSS threshold	Jonkman and Markou (2006)
Reduced amygdala central nucleus DA	Mec-precipitated somatically expressed withdrawal behaviors	Panagis et al. (2000)
Increased α7-mediated NE released	Hippocampal slice from rats in spontaneous withdrawal	Barik and Wonnacott (2006)
Increased hypothalamic NE levels and utilization	Spontaneous withdrawal from cigarette smoking	Andersson et al. (1989)
Reduced NAcc DA output/increased clearance	Spontaneous withdrawal from nicotine self-administration	Rahman et al. (2004)
Abolished effect of nicotine on limbic DA metabolism	Spontaneous withdrawal	Salminen et al. (1999)
Decreased DA output and metabolites in NAcc (not in frontal cortex)	Mec-precipitated somatically expressed withdrawal behaviors	Hildebrand et al. (1998)
Decreased NAcc striatum DA content	Spontaneous withdrawal, reduced locomotor activity	Fung et al. (1996)
Reduced number of D2 receptors	Spontaneous withdrawal, reduced locomotor activity	Fung et al. (1996)
GABAergic mechanisms		
Increased diazepam binding inhibitor mRNA	Spontaneous withdrawal in mice	Kutsura et al. (2001)
GABA-B agonist does not affect nicotine withdrawal	ICSS thresholds	Patterson et al. (2005)
Endocannobioids		
D9-tetrahydrocannobinol decreased mec- and naloxone-precipitated withdrawal	Somatically expressed withdrawal behaviors and condition place aversion	Balerio et al. (2004)

(continued)

Table 3 (continued)

CB1 receptor knock-out mice	No change in mec-precipitated somatically expressed withdrawal signs	Castañé et al. (2002)
Endogenous opiate peptides		
Naloxone precipitates nicotine withdrawal thresholds	Somatically expressed withdrawal behaviors	Adams and Cicero (1998); Carboni et al. (2000); Malin et al. (1993)
Naloxone precipitates nicotine withdrawal	Somatically expressed withdrawal behaviors, but not ICSS thresholds	Watkins et al. (2000)
Morphine potently reverse nicotine withdrawal	Somatically expressed withdrawal behaviors	Malin et al. (1993)
Naloxone precipitates nicotine withdrawal in mice	Somatically expressed withdrawal behaviors	Biala et al. (2005)
Naloxone induces place aversion in nicotine-dependent rats	Conditioned place aversion	Ise et al. (2000); Watkins et al. (2000)
Naloxone prevents nicotine alleviation of nic. withdrawal	Somatically expressed withdrawal behaviors	Malin et al. 1996
Mu and delta agonists reduced mec-precipitated aversion	Conditioned place aversion	Ise et al. (2000)
Kappa antagonist suppresses mec.-precipitated aversion	Conditioned place aversion	Ise et al. (2000)
β-Endorphin metabolite Gly-Glu blocks aversiveness of mec-precipitated withdrawal	Conditioned place aversion	Göktalay et al. 2006
Mec precipitates hyperalgesia in nicotine-tolerant rats	Nociceptive jaw-opening reflex	Schmidt et al. (2001)
NAcc Met-enkephalin increased	Spontaneous withdrawal in mice	Isola et al. (2002)
Striatum preproenkephalin mRNA increased	Spontaneous withdrawal in mice	Isola et al. (2002)
Attenuated withdrawal in μ-opiod receptor knockout mice	Somatically expressed withdrawal behaviors in mice	Berrendero et al. (2002)
Attenuated withdrawal in preproenkephalin knockout mice	Somatically expressed withdrawal behaviors in mice	Berrendero et al. (2005)
Analog of anti-opiod peptide precipitates nicotine withdrawal	Somatically expressed withdrawal behaviors	Malin et al. (1996b)
Excitatory amino acids		
mGlu5R antagonist increases withdrawal signs	Somatically expressed withdrawal behaviors and ICSS thresholds	Liechti and Markou (2007)
mGlu2/3R antagonist reduces the effect of the mGlu5R antagonist	ICSS thresholds	Liechti and Markou (2007)
mGlu2R agonist (systemic and VTA) precipitated withdrawal	ICSS thresholds	Kenny et al. (2003)
mGlu2R antagonist reduces spontaneous withdrawal signs	ICSS thresholds	Kenny et al. (2003)
AMPA antagonist precipitates withdrawal signs	ICSS thresholds	Kenny et al. (2003)

while the firing rate of substantia nigra dopamine neurons significantly increased. It is possible that the differing infusion procedures in these two studies may have resulted in a different time-course of electrophysiological events. It would be of interest to compare the time-course these cellular adaptations with that of nicotine withdrawal signs.

Carboni et al. (2000) reported increased DA output in the prefrontal cortex during mecamylamine-precipitated withdrawal. The striatum appears to become insensitive to nicotine during withdrawal, as indicated by loss of the cFos response to nicotine. This was also true of a limbic region, the cingulate gyrus (Salminen et al. 1999).

The amygdala, a region that mediates many aversive responses, is activated during nicotine withdrawal, as indicated by an increase in Fos-positive cells (Panagis et al. 2000). There is reduced amygdala DA output during withdrawal (Panagis et al. 2000). Also, the light avoidance response, a putative indicator of anxiety during nicotine withdrawal, is largely eliminated by injection of the serotonergic drug odansetron into the amygdala (Costall et al. 1990b). The injection of odansetron into the dorsal raphe' nucleus (DRN), a major center of serotonergic neurons, also greatly reduces light avoidance during nicotine withdrawal (Costall et al. 1990b). Reduced social interaction during nicotine withdrawal, another indicator of anxiety, was restored by direct injection of nicotine into the DRN (Cheeta et al. 2001). In summary, the diversity of brain anatomical regions affected by nicotine withdrawal might help account for the diversity of withdrawal symptoms during smoking cessation.

5 Neurochemical Correlates

Nicotinic cholinergic receptors are located on cells that release a wide variety of transmitters (see chapter by Barik and Wonnacott, in this volume), so that nicotine interacts with multiple neurochemical pathways. The roles of cholinergic, dopaminergic, and endogenous opioid systems in physical dependence and withdrawal have been most thoroughly studied and documented. Research on the role of other transmitters and neurochemical mechanisms is rather scattered. Overall, however, research with rodent models of physical dependence has provided a wealth of potential targets for experimental treatments to aid smoking cessation.

5.1 Cholinergic Mechanisms

Since nicotine is a cholinergic drug, it is a foregone conclusion that cholinergic mechanisms are involved in nicotine physical dependence and withdrawal. For example, tritiated nicotine binding in mouse cortex and midbrain increased markedly during the first few days of withdrawal from weeks of chronic nicotine in drinking water (Pietilä et al. 1998). Subtypes of nicotinic cholinergic receptors are described in the chapters by Collins et al. and Barik and Wonnacott, in this volume.

Several studies have suggested the involvement of specific nicotinic cholinergic receptor subtypes. This is potentially important, since it might guide more specifically targeted nicotine replacement therapies. Nicotine reinforcement and self-administration prominently involves α4β2 receptors (Picciotto et al. 1998). It is possible that these receptors may also be involved in nicotine withdrawal syndrome. The competitive nicotinic receptor antagonist DHβE has relative specificity for this class of receptor. Peripherally administered DHβE precipitated nicotine withdrawal, as indicated by raised ICSS thresholds, while centrally administered DHβE precipitated nicotine withdrawal, as indicated by somatically expressed behavioral signs (Epping-Jordan et al. 1998; Malin et al. 1998b). However, knockout mice devoid of β2 subunits still demonstrated somatically expressed nicotine withdrawal behaviors (Besson et al. 2006; Salas et al. 2004). In contrast, genetic deletion of the β4 subunit eliminated somatically expressed nicotine withdrawal behaviors in the mouse (Salas et al. 2004). Centrally administered hexamethonium, a noncompetitive nicotinic receptor antagonist with relative specificity for α3β4 receptors, precipitated somatically expressed nicotine withdrawal signs with enormous potency (Malin et al. 1997), particularly as compared with DHβE (Malin et al. 1998b). One speculative possibility is that α4β2 receptors, with their close connection to reinforcement mechanisms, might be responsible for withdrawal-induced changes in those mechanisms, while α3β4 receptors might be involved in the heightened irritability leading to somatically expressed nicotine withdrawal signs.

The pattern of data on the role of α7 receptors (pentamers of the α7 subunit) is far from clear. Nomikos et al. (1999) reported sharply reduced locomotor activity in nicotine-dependent rats injected with the selective α7 antagonist methyllycaconitine (MLA). Barik and Wonnacott (2006) found increased α7 sensitivity in the hippocampus of rats during nicotine withdrawal, as evidence by increased norepinephrine release in response to an α7 agonist. On the other hand, Markou and Paterson (2001) reported that systemically administered MLA failed to precipitate either somatically expressed withdrawal behaviors or altered ICSS thresholds.

There have been few studies of acetylcholine release during nicotine withdrawal. Rada et al. (2001) found that withdrawal from continuous nicotine infusion resulted in significantly increased acetylcholine release in the NAcc, concomitant with somatically expressed withdrawal behaviors. This was similar to the effect of morphine withdrawal on NAcc acetylcholine release. Since dopamine release was decreased at the same time, the transmitter balance in the NAcc was radically altered.

5.2 Dopaminergic Mechanisms

It is well established that nicotine stimulation of the mesolimbic dopamine (DA) pathway is essential to the reinforcing action of nicotine (Balfour 2004; Corrigall and Coen 1991). Considerable evidence suggests that alterations in this pathway may also be essential to the nicotine abstinence syndrome, particularly to its depression-like dimension. During withdrawal from continuous nicotine infusion, rats displayed reduced activity levels and reduced DA content in the striatum

and NAcc as well as reduced D2 DA receptors in the NAcc (Fung et al. 1996). A particularly consistent finding is lowered DA output in the NAcc during nicotine withdrawal in rodent models, with a time course roughly consistent with various behavioral withdrawal signs (Carboni et al. 2000; Hildebrand et al. 1998; Lindblom et al. 2005; Rada et al. 2001; Rahman et al. 2004). This decreased release is likely due to alterations at the origin of the mesolimbic DA pathway in the VTA, since mecamylamine injected directly into the VTA, but not the NAcc, of nicotine-dependent rats triggered both withdrawal signs and reduced NAcc DA output (Hildebrand et al. 1999).

It has long been established that mesolimbic dopamine activity is essential to intracranial self-stimulation (Zarevics and Setler 1979). Thus, alterations in NAcc DA function may underlie the increased NAcc ICSS thresholds that serve as a laboratory model of the anhedonia that characterizes depression (Epping-Jordan et al. 1998). This is supported by recent research suggesting that the ability of bupropion to reverse withdrawal-induced elevation of ICSS thresholds is closely associated with its ability to restore mesolimbic DA release (Paterson et al. 2007). However, dopaminergic effects may not necessarily underlie the somatically expressed withdrawal behaviors. The opiate antagonist naloxone precipitates those signs in nicotine-dependent rodents, but neither decreased NAcc DA output (Carboni et al. 2000) nor increased ICSS thresholds (Watkins et al. 2000). There may be a related anatomical dissociation. Nicotinic or dopaminergic antagonists injected directly in the amygdala of nicotine dependent rats fail to increase ICSS thresholds (Jonkman and Markou 2006). However, mecamylamine-precipitated nicotine withdrawal caused reduced DA output in the central nucleus of the amygdala, along with somatically expressed withdrawal behaviors (Panagis et al. 2000). The central nucleus of the amygdala is implicated in control of anxiety reactions and response to stressors (Bohus et al. 1996), so dopaminergic alterations there may be connected to the anxiety/irritability dimension of nicotine withdrawal syndrome.

5.3 Endogenous Opiate Mechanisms

The major somatically expressed nicotine withdrawal signs in the rat (gasps/writhes, shakes/tremors, teeth chatter/vacuous chewing, ptosis, scratches, and spontaneous ejaculation are all routinely observed in mild to moderate opiate abstinence syndrome (Malin et al. 1990). Jumping is a prominent morphine withdrawal signs in mice, but not in rats, and the same is true in nicotine withdrawal (Isola et al. 1999; Malin et al. 1992; Semenova et al. 2003). This raised the question of whether nicotine withdrawal syndrome and opiate withdrawal syndrome reflected similar underlying states of the organism. Nicotine induces the release of enkephalins and beta-endorphin (Gilbert et al. 1992; Suh et al. 1995). Therefore, prolonged nicotine exposure would be expected to cause prolonged overstimulation of opiate receptors. The sudden termination of nicotine exposure might then lead to reduced stimulation

of opiate receptors, possibly inducing an opiate abstinence-like state. There is now extensive evidence supporting this hypothesis, at least in terms of somatically expressed withdrawal behaviors and withdrawal-induced hyperalgesia. Morphine reverses the somatically expressed withdrawal syndrome, even at subanalgesic doses (Malin et al. 1994). It also potently reverses nicotine withdrawal-induced hyperalgesia (Schmidt et al. 2001). There have been many reports that the opiate antagonist naloxone precipitates somatically expressed nicotine abstinence syndrome in chronically nicotine-treated rats or mice (Adams and Cicero 1998; Biala et al. 2005; Carboni et al. 2000; Malin et al. 1993a; Watkins et al. 2000). Naloxone-precipitated nicotine withdrawal is highly aversive, as indicated by conditioned place preference (Ise et al. 2000; Watkins et al. 2000), although it does not raise ICSS thresholds (Watkins et al. 2000).

Naloxone binds to a variety of opiate receptor subtypes. Selective mu- and delta-opiate receptor antagonists precipitated conditioned place aversion in nicotine-dependent rats (Ise et al. 2000). The nonopioid peptide, neuropeptide FF (NPFF), has potent antiopiate actions and may play a role in opiate dependence (Malin et al. 1990). Systemic injection of a systemically active NPFF analog precipitated a withdrawal syndrome in morphine-dependent rats (Malin et al. 1993b) and also in nicotine-dependent rats (Malin et al. 1996b). There are opposing interactions between kappa-opiate receptors and other opiate receptors. Alterations in the balance between these opposing opiate systems may contribute to morphine dependence and abstinence syndrome (Narita et al. 2001). Therefore, it is consistent with the endogenous opioid hypothesis of nicotine dependence, that a kappa-opiate receptor antagonist reduced the aversiveness of mecamylamine-precipitated nicotine withdrawal (Ise et al. 2002). The beta-endorphin metabolite Gly–Gln also reduced the aversiveness of precipitated nicotine withdrawal (Göktalay 2006).

Nicotine injection immediately reverses somatically expressed nicotine withdrawal syndrome (Malin et al. 1992), but it fails to do so after pretreatment with naloxone to block opiate receptors (Malin et al. 1996a). This suggests that nicotine relieves this aspect of nicotine withdrawal syndrome through inducing renewed release of endogenous opioid peptides.

Genetically engineered mouse strains have been used to further assess the role of endogenous opioid mechanisms. There was a significantly attenuated nicotine abstinence syndrome in knockout mice lacking the gene for mu-opiate receptors (Berrendero et al. 2002) as well as the gene for the enkephalin precursor preproenkephalin (Berrendero et al. 2005). Isola et al. (2002) observed changes in brain opiate peptides during nicotine withdrawal in the mouse. Met-enkephalin concentration increased in the nucleus accumbens, and preproenkephalin mRNA increased in the striatal region as a whole. Finally, several of the signal transduction processes implicated in nicotine dependence are also involved in opiate dependence, as discussed below. Overall, there is extensive evidence that endogenous opiate mechanisms contribute to nicotine dependence and at least certain features of nicotine withdrawal syndrome, while the additional biochemical factors discussed in other subsections of Sect. 5 may correspond to some of the differences between nicotine and opiate dependence.

5.4 Serotonin and Other Transmitters

Various serotonergic mechanisms may play complex or even opposing roles in nicotine withdrawal syndrome. Acute nicotine administration increased serotonin release in the brain (Ribeiro et al. 1993), while spontaneous nicotine withdrawal reduced serotonin turnover in whole brain (Yasuda et al. 2002). Conversely, the serotonin precursor 5-HTP reversed withdrawal-induced immobility in the forced swim test, a putative model of depression (Mannucci et al. 2006). Also, stimulation of 5HT3 receptors may attenuate various features of nicotine withdrawal syndrome such as anxiety, as indicated by the light avoidance test (Costall et al. 1990a, b), or aversiveness, as indicated by conditioned place aversion (Suzuki et al. 1997). These results might suggest that serotonergic hypoactivity may contribute to nicotine abstinence syndrome, while renewed serotonin activity may moderate the syndrome. Stimulation of 5-HT1A receptors, on the other hand, may either contribute to or attenuate certain features of nicotine withdrawal syndrome. Numbers of diencephalic 5-HT1A receptors were reduced following induction of nicotine dependence and at 15 and 30 days following nicotine withdrawal (Mannucci et al. 2006). A selective 5-HT1A antagonist reduced withdrawal-induced increases in startle response (Rasmussen et al. 2000). A 5HT-1A antagonist combined with the serotonin reuptake inhibitor fluoxetine reversed the withdrawal-induced increases in ICSS thresholds (Harrison et al. 2001). In contrast, a 5-HT1A antagonist reversed the apparent antidepressant-like effects of serotonin precursor loading in nicotine-abstinent mice undergoing the forced swim test. In interpreting these results, it must be remembered that many, but not all, 5-HT1A receptors are serotonergic autoreceptors whose activation reduces serotonin release (Guilloux et al. 2006). Finally, stimulation of 5-HT2 receptors may contribute to certain somatically induced behavioral signs. Nicotine withdrawal resulted to increased sensitivity to induction of wet-dog shakes and head twitches by a selective 5-HT2 agonist (Suemaru et al. 2001; Yasuda et al. 2002).

Despite the long-established ability of nicotine to release norepinephrine (NE) from the sympathetic nervous system, there has been surprisingly little study of NE in nicotine withdrawal. Alpha-7 nicotinic receptors positively regulate NE release in hippocampal slices, and this effect was augmented in slices obtained from rats undergoing nicotine withdrawal (Barik and Wonnacott 2006). In an early study, there were increased NE levels and NE utilization in several hypothalamic regions in rats undergoing withdrawal from chronic exposure to cigarette smoke (Andersson et al. 1989).

Plasticity of excitatory amino acid mechanisms, critical for learning and log-term potentiation, is involved in acquiring dependence on a number of drugs (Siggins et al. 2003). Nicotine dependence is no exception, based on the effects of various selective glutamate agonists and antagonists on rat continuous infusion models. Stimulation of several excitatory glutamate receptors (AMPA receptors and metabotropic mGlu5Rs) appears to prevent or attenuate nicotine withdrawal syndrome (Kenny et al. 2003; Liechti and Markou 2007). Conversely, stimulation of metabotropic mGlu2/3Rs, often involved in presynaptic inhibition of glutamate re-

lease, appears to intensify nicotine abstinence syndrome, as indicated by changes in ICSS thresholds (Kenny et al. 2003; Liechti and Markou 2007).

There has been little published research on GABAergic mechanisms in nicotine physical dependence and withdrawal syndrome. A GABA-B agonist did not affect the withdrawal-induced increases in ICSS thresholds (Paterson et al. 2005). However, the message for diazepam binding inhibitor, a potent modulator of GABA-A receptors, increased significantly during spontaneous withdrawal in mice (Katsura et al. 2001). This suggests that the role of GABA-A receptor complex should be further explored.

The endocannabinoid system might also be involved, since Δ9-tetrahydorcannabinol, the major active ingredient in cannabis, decreases somatically expressed withdrawal behaviors and the aversiveness of withdrawal in mecamylamine- and naloxone-precipitated nicotine abstinence (Balfour 2002). However, genetic knockout of the CB1 cannabinoid receptors did not significantly affect somatically expressed withdrawal behaviors (Castañé et al. 2002).

Preliminary evidence suggests that a variety of nonopioid peptides may modulate nicotine dependence and withdrawal syndrome. For example, genetic knockout of calcitonin gene-related peptide largely eliminated somatically expressed withdrawal behaviors (Salmon et al. 2004). An antagonist against corticotropin releasing factor (CRF) prevented increased ICSS thresholds in mecamylamine-precipitated, but not in spontaneous nicotine withdrawal (Bruijnzeel et al. 2007). Altered responsiveness to neuropeptide Y in the paraventricular area may be relevant to withdrawal-induced changes in appetite and weight gain (Bishop et al. 2002). Finally, there is preliminary evidence of hormonal correlates of withdrawal, including altered corticosteroid stress response (Semba et al. 2004) and changes in brain and plasma concentrations of various steroids, such as progesterone (Concas et al. 2006). As with anatomical correlates of nicotine withdrawal syndrome, the diversity of neurochemical pathways involved may help account for the diversity of withdrawal symptoms during smoking cessation.

5.5 Signal Transduction Mechanisms

Opiate abstinence syndrome is characterized by increases in cyclic adenosine monophosphate (cAMP) and nitric oxide synthesis. Some similar phenomena appear in nicotine withdrawal, underscoring the putative role of endogenous opioid peptides. cAMP is upregulated in the amygdala, possibly contributing to increased anxiety during nicotine withdrawal (Tzavara et al. 2002). Chronic nicotine exposure increases nitric oxide metabolites in multiple brain regions (Pogun et al. 2000), and there is persistently increased hypothalamic nitric oxide response to food deprivation following nicotine withdrawal (Mannucci et al. 2005). Several inhibitors of nitric oxide synthase prevent mecamylamine-precipitated nicotine withdrawal syndrome and reverse spontaneous withdrawal syndrome, as indicated by somatically expressed behaviors (Adams and Cicero 1998; Malin et al. 1998b). Finally,

ion channel events are also likely to participate in the expression of nicotine withdrawal syndrome. An antagonist against L-type voltage-regulated calcium channels attenuated mecamylamine-precipitated somatically expressed withdrawal behaviors (Biala and Weglinska 2005).

5.6 Gene Expression

The extensive adaptations underlying drug dependence probably require changes in gene expression. The immediate early gene c-fos is a marker for acute cellular activation. Its expression is rapidly induced in the central nucleus of the amygdala in mecamylamine-precipitated withdrawal, simultaneous with somatically expressed signs (Panagis et al. 2000), consistent with a rapid induction of anxiety. An early event in spontaneous withdrawal is increased message for brain-derived neurotrophic factor (BDNF) in the hippocampus (Kenny et al. 2000). The transcription factor ΔFosB, implicated in stimulant drug dependence, is selectively increased in the nucleus accumbens during spontaneous nicotine withdrawal (Marttila et al. 2006). In contrast, there is decreased cortical DNA binding of the transcription factor AP-1 during spontaneous withdrawal (Pandey et al. 1999). During nicotine withdrawal, there are also decreases in the cAMP-responsive transduction factor phospho-CREB in the neocortex, paleocortex, and nucleus accumbens (Pandey et al. 2001; Pluzarev and Pandey 2004). There is also decreased binding to the genomic target of CREB, CRE-DNA (Pandey et al. 2001). Consistent with these alterations in various transcription factors, microarray studies have documented multiple region-specific changes in gene expression during prolonged nicotine exposure (Konu et al. 2001).

6 Developmental Factors

Early adolescent smoking onset is a risk factor for severe addiction and difficulty in smoking cessation in adulthood (Breslau and Peterson 1996). Therefore, it is significant that nicotine has more potent rewarding effects in adolescent as opposed to adult rodents (Adriani et al. 2004; Belluzzi et al. 2004; Vastola et al. 2002). However, several studies suggest that adolescent rodents develop physical nicotine dependence less readily than adults, as indicated by less severe nicotine withdrawal syndrome. Unlike adult rats, continuously nicotine-infused adolescent rats failed to display mecamylamine-precipitated withdrawal behaviors (O'Dell et al. 2004, 2006). They also displayed less mecamylamine-precipitated conditioned place aversion than adults (O'Dell et al. 2007). In addition, nicotine-infused adolescent rats experienced smaller mecamylamine-precipitated ICSS threshold elevations than adults (Kota et al. 2007). Nicotine-infused adolescent mice also displayed fewer spontaneous and mecamylamine-precipitated somatically expressed withdrawal behaviors

than did adults (Kota et al. 2007). Consistent with these results, Wilmouth and Spear (2006) found that adult, but not adolescent rats developed heightened startle response (indicative of irritability or anxiety) during spontaneous withdrawal following continuous nicotine infusion. However, adolescent, but not adult, rats displayed disrupted prepulse inhibition during spontaneous withdrawal, indicative of impaired selective attention. This result raises the possibility that adolescent nicotine withdrawal might primarily involve cognitive disruption. It may be of interest to investigate longer-term or delayed consequences of adolescent nicotine exposure, since nicotine-induced changes in gene expression reach a peak around the time of puberty (Polesskaya et al. 2007).

7 Evaluating Potential Therapies

Research with rat continuous infusion models established that such models are sensitive to putative rodent counterparts (Cryan et al. 2003; Malin et al. 2006) of the clinical benefits of bupropion in alleviating withdrawal signs and symptoms in smoking cessation (Shiffman et al. 2000). A number of newer experimental medications or lead compounds for potential medications have been evaluated for effects on rodent models of nicotine withdrawal syndrome. For example, the $\alpha 4\beta 2$ nicotinic receptor partial agonist SSR591813 reduced somatically expressed withdrawal behaviors (Cohen et al. 2003). As noted above, the 5-HT3 agonist odansetron and several 5-HT1A antagonists alleviated various measures of nicotine withdrawal syndrome in rodent models (Costall et al. 1990a, b; Harrison et al. 2001; Rasmussen et al. 2000; Suzuki et al. 1997). The serotonin/dopamine antagonist clozapine, commonly employed as an antipsychotic agent, prevented somatically expressed spontaneous withdrawal behaviors, while its effect on ICSS thresholds depended on dosing regimen and initial responsiveness to clozapine (Semenova and Markou 2003). A metabotropic glutamate receptor II antagonist prevented ICSS threshold elevation in spontaneous withdrawal (Kenny et al. 2003; Liechti and Markou 2007; Markou 2007). The norepinephrine reuptake inhibitor atomoxetine, commonly employed as a treatment for attention deficit disorder, reversed the impairment in contextual fear conditioning in spontaneous nicotine withdrawal (Davis and Gould 2007). This suggests a novel approach to treating cognitive deficits in smoking cessation.

Several natural products have been evaluated in rodent models of nicotine withdrawal. An extract of *Hypericum perforatum* (St. John's Wort, a putative antidepressant, and inhibitor of serotonin reuptake) reversed somatically expressed withdrawal behaviors and locomotor depression in spontaneous withdrawal (Catania et al. 2003). A benzoflavone compound isolated from *Passiflora incarnata*, interfered with the induction of physical dependence. Coadministration with chronic nicotine prevented various subsequent indicators of withdrawal syndrome in the mouse, including jumping, locomotor inactivity, immobility in the swim test and naloxone-precipitated escape jumping (Dhawan et al. 2002).

Another approach to experimental treatment for smoking cessation is immunization against nicotine. While nicotine by itself is a small, nonimmunogenic molecule, it can be rendered immunogenic by conjugating it to a large carrier protein (Pentel et al. 2000). Antibodies raised against such conjugated immunogens delay nicotine entry into the central nervous system and attenuate nicotine's stimulus properties and several of its behavioral and physiological effects (Malin et al. 2002; Pentel et al. 2000). Passive immunization with antibodies against nicotine prevents nicotine injection from relieving nicotine withdrawal syndrome in the rat, as indicated by somatically expressed withdrawal behaviors (Malin et al. 2001). This suggests that an immunized smoker undergoing the discomforts of smoking cessation might not be able to find relief through renewed smoking, thus removing one cause of early relapse. Surprisingly, Lindblom et al. (2005) found that actively immunized rats experienced a milder nicotine withdrawal syndrome, in terms of somatically expressed signs and alterations of dopamine release in the nucleus accumbens. One possible explanation for this is that nicotine antibodies cause a more gradual elimination of nicotine from the body (Keyler et al. 1999).

A number of other preclinical studies cannot be described here due to proprietary restrictions. Clearly, however, the assessment of drug effects on rodent models of nicotine physical dependence and withdrawal syndrome is becoming an increasingly standard procedure in screening potential therapies for smoking cessation.

8 Directions for Future Research

Laboratory research on nicotine physical dependence has expanded in many directions since the introduction of rodent models in the early 1990s. However, it is not difficult to detect a number of gaps in our knowledge. For example, while various versions of rodent models reproduce most features of the smoking cessation withdrawal syndrome, little has been published regarding sleep disturbances. Also, it might be interesting to examine other measures of physical dependence beyond the severity of the withdrawal syndrome. One alternate approach to measuring dependence might be to determine the minimum dose of nicotinic antagonist necessary to precipitate a threshold degree of withdrawal signs.

In order to increase the external validity of our models, it might be desirable to consider some nonnicotine ingredients of tobacco smoke. It appears that there are natural monamine oxidase (MAO) inhibitors in tobacco smoke (Lewis et al. 2007). It would be interesting to determine whether the coadministration of a low dose of a standard MAO inhibitor along with chronic nicotine would increase physical dependence, as assessed by various withdrawal measures. In view of the antidepressant properties of MAO inhibitors, measures reflecting aspects of depression might be particularly affected.

Studies of brain mechanisms have tended to focus primarily on structures associated with the mesolimbic DA pathway. Ideally, all brain regions with major concentrations of nicotinic acetylcholine receptors should be probed with nicotinic

antagonists to determine all sites where various dimensions of withdrawal syndrome can be precipitated. Advances in imaging techniques, such as fMRI, might also help identify critical structures and systems in inducing dependence and expressing withdrawal. Also, surprisingly little has been done to observe alterations in cellular or regional electrophysiological activity during nicotine withdrawal. Very little has been published on sex differences or the effect of the estrous cycle in rodent models of physical dependence. For example, it would be interesting to see if male animals and female animals at different estrous phases share the same pattern of relative intensity among measures reflecting the various dimensions of withdrawal syndrome (irritability, anxiety, depression etc.). At the receptor level, it would be interesting to determine whether dependence induction depends on the stimulation or the desensitization of nicotinic receptors by chronic drug exposure.

In recent years, several effective medications have been introduced to aid smoking cessation. Yet no one treatment has consistently reached a long-term quit rate of over 50%. One strategy to break through this barrier might be to evaluate combinations of treatments in preclinical models. It is noteworthy that both of the effective nonnicotine medications, bupropion and varenicline, show probable activity against withdrawal syndrome as well as against the positive reinforcing effects of tobacco products. Therefore, efforts to find better interventions against nicotine physical dependence and withdrawal syndrome should be an integral element of any comprehensive research and development program to aid smoking cessation.

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Part IV
Nicotine and Tobacco Product Regulation

Approaches, Challenges, and Experience in Assessing Free Nicotine

David L. Ashley, James F. Pankow, Ameer D. Tavakoli, and Clifford H. Watson

Contents

Abstract Delivery of nicotine in the most desirable form is critical in maintaining people's use of tobacco products. Interpretation of results by tobacco industry scientists, studies that measure free-base nicotine directly in tobacco smoke, and the variability of free-base nicotine in smokeless tobacco products all indicate that the form of nicotine delivered to the tobacco user, in addition to the total amount, is an important factor in whether people continue to use the product following their initial exposure. The physiological impact of nicotine varies with the fraction that is in the free-base form and this leads to continued exposure to other toxic tobacco contents

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and emissions. In addition to evaluating the total nicotine delivered to the user, measuring the fraction of nicotine in the free-base form is critical in understanding and controlling the influence of nicotine on tobacco use.

1 Introduction

The acid–base chemistry of nicotine is now well known and investigations have shown that nicotine in tobacco smoke or in smokeless tobacco products can exist in pH-dependent protonated or unprotonated free-base forms. In tobacco smoke, only the free-base form can volatilize readily from the smoke particulate matter to the gas phase, with rapid deposition in the respiratory tract. Using volatility-based analytical measurements, the fraction of nicotine present as the free-base form can be quantitatively determined. For smokeless tobacco products, the situation differs because the tobacco is placed directly in the oral cavity. Hence, the pH of smokeless tobacco products can be measured directly to yield information on the fraction of nicotine available in the unprotonated free-base form. It is important to characterize the fraction of total nicotine in its conjugate acid–base states as this dramatically affects nicotine bioavailability, because the protonated form is hydrophilic while the unprotonated free-base form is lipophilic and thus readily diffuses across membranes (Armitage and Turner 1970; Schievelbein et al. 1973). As drug delivery rate and addiction potential are linked (Henningfield and Keenan 1993), increases in delivery rate due to increased free-base levels affect the addiction potential.

As in any attempt to deliver a drug by smoking, two steps are involved in nicotine delivery: step 1, drug transfer from the tobacco filler to smoke; and step 2, drug transfer from the smoke to the user. For a specific nicotine quantity in a cigarette's rod material, increasing the efficiency of either or both transfer steps will increase total delivery to the smoker. The amount of nicotine or other smoke constituent delivered to a human (or to a machine used to simulate human smoking) is frequently expressed as units of amount per cigarette, i.e., mg nicotine per cigarette.

Because of nicotine's addictive nature, factors altering its overall delivery and delivery rate are crucial in understanding how tobacco products influence their continued use. The tobacco industry has conducted over 40 years of research on both steps 1 and 2 of the cigarette-to-smoker nicotine transfer. Numerous reports, memos, and letters concerning this research (as well as a wide range of other topics) were acquired during litigation against the industry, and have been archived within the digital Legacy Tobacco Documents Library (http://legacy.library.ucsf.edu/). Some important examples of the research on steps 1 and 2 are discussed by Henningfield et al. (2003), as well as later in this chapter. Particularly interesting is research involving the effects of numerous additives, including basic additives, on the two transfer efficiencies. In addition, recent work that directly measures the free-base nicotine levels in tobacco smoke particulate has provided interesting findings about the influence of cigarette design features on nicotine's conjugate acid–base forms present in cigarette smoke. Finally, a series of investigations has

shown substantial variation in the pH of smokeless tobacco products when examining a wide range of such products, suggesting that manufacturers carefully address the users' nicotine delivery needs. These areas of investigation provide insight into why tobacco products continue to be widely used despite the associated high morbidity and mortality.

1.1 Alkaloid Chemistry

Many pharmacologically active organic chemicals found in nature are alkaloids. In general, these compounds contain one or more nitrogen atoms, which in turn impart some basicity to the molecule. Well-known alkaloid examples are caffeine, cocaine, codeine, ephedrine, morphine, nicotine, quinine, and scopolamine. Heroin is derived from morphine by a chemical modification that increases lipophilicity, making the heroin molecule inherently more pharmacologically potent than morphine. The exhibition of its basic properties by an alkaloid (Alk) involves (by definition) the acceptance of a proton H^+ according to:

$$\underset{\substack{\text{free base}\\\text{(volatilizable)}}}{\text{Alk}} + \underset{\text{proton}}{H^+} = \underset{\substack{\text{mono-protonated}\\\text{(not volatilizable)}}}{AlkH^+}. \quad (1)$$

Relative to the conjugate acid $AlkH^+$, the conjugate base Alk is *free* of the H^+. Hence, Alk is frequently referred to as the "free-base" form of the alkaloid. Reaction 1 occurs readily in many environments including cellular cytoplasm, water, the particulate matter droplets of tobacco smoke, and blood serum.

Like H^+, the chemical ion $AlkH^+$ carries unit positive charge. To maintain charge neutrality, each positive ion must be accompanied by a corresponding anionic (negative) charge, e.g., the chloride ion Cl^-, an organic anion such as acetate CH_3COO^-, or some other anion. Reaction 1 can be rewritten to acknowledge the accompanying presence of an anionic charge-carrying species. With Cl^- as the anion, the resulting chemical equation becomes:

$$\underset{\substack{\text{free base}\\\text{(volatilizable)}}}{\text{Alk}} + \underset{\text{proton}}{H^+} + \underset{\text{anion}}{Cl^-} = \underset{\substack{\text{mono-protonated}\\\text{(not volatilizable)}}}{AlkH^+} + \underset{\text{anion}}{Cl^-} \quad (2)$$

thereby demonstrating that protonation of an alkaloid occurs via reaction of Alk with an acid (H^+Cl^- in this example). As discussed by Pankow (2001), the term "free-base" has become an important and common property descriptor of the alkaloid's conjugate base form (Alk) because loss of the H^+ also brings freedom from the powerful electrostatic attraction forces from the surrounding negative charge. Consequently, as compared to the conjugate acid $AlkH^+$ form, which is essentially nonvolatile, the Alk free-base form possesses much higher volatility, and has a much higher propensity for entering the gas phase.

For the alkaloid drug cocaine, the free-base form can be represented as Coc. Powdered "street cocaine" is usually cocaine in combination with the acid H^+Cl^-, so we represent street cocaine as $CocH^+Cl^-$. This protonated (conjugate acid) form of cocaine contains strong ionic forces that preclude smoking as an effective delivery means. Strong ionic forces present in the conjugate acid results in it having minimal volatility with very inefficient transfer to smoke. Street cocaine users require other efficient administration techniques, e.g., "snorting" (insufulation) or intravenous injection. However, if a base (e.g., grocery-store sodium bicarbonate or ammonia) is used in "free-basing", the conjugate acid $CocH^+Cl^-$ form can be converted to volatile free-base form (Coc). "Crack" cocaine, as an impure form of free-base cocaine, is often smoked. Crack poses such a big problem in contemporary society because of: (i) its great ease of preparation; (ii) the pharmacological potency of cocaine; and (iii) the rapidity of delivery to the brain, made possible by inhaling the drug into lung tissues (i.e., for smoking the path is lungs → heart → brain, while for administration by nonsmoking means the path is: systemic circulation → heart → lungs → heart → brain).

1.2 Free-Base Nicotine

Regarding the first transfer step from product to smoke, basic additives assist in the formation of nicotine in its free-base form, promoting volatilization from the burning tobacco to the mainstream cigarette smoke. In recent years, industry scientists (e.g., Seeman et al. 1999) have published studies in the open literature arguing that a portion of nicotine in tobacco volatilizes without the benefit of additives. With most nicotine in tobacco being associated with organic acids (e.g., citric acid), which are far weaker acids than the H^+Cl^- found in street cocaine, this is certainly true. And, of course, centuries of tobacco smoking by indigenous peoples of the Americas dating to pre-Columbian times (Wilbert 1987) obviously attest to this conclusion. However, the fact remains that numerous industry studies have investigated and demonstrated the efficacy of *increasing* the nicotine transfer efficiency of step 1 by means of basic additives. Moreover, Henningfield et al. (2003) discuss data within the Seeman et al. (1999) study, supporting the view that basic additives can increase the nicotine transfer efficiency of step 1.

Regarding step 2, the transfer of nicotine from smoke to the user, an example of the long-standing interest of the tobacco industries in nicotine uptake (and free-base nicotine in particular) from tobacco smoke is a 1967 British American Tobacco Co. report on experiments involving dogs sacrificed for their lungs, which were then connected to a smoking machine and an artificial heart (Evelyn 1967). Demonstrating their interest in the issue, industry-funded scientists found that, as tobacco smoke of various types was introduced to the lungs, nicotine levels increased in the artificial "blood." In terms of knowledge regarding nicotine deposition in the lungs, Pankow (2001) discusses that: (i) currently available evidence is consistent with the view that most of the total nicotine in fresh mainstream tobacco smoke resides in the

smoke particulate matter (PM); and (ii) if all mainstream smoke nicotine were in the gas phase, very little would reach the lungs, depositing rather in the mouth and upper respiratory tract. Consequently, nicotine's volatility from tobacco smoke particles is of special importance given that: (i) the deposition efficiency for tobacco smoke particles $F^{\text{particles}}$ (see Pankow 2001 for other related notation) has been measured to be as low as 40–60% (Hinds et al. 1983), while (ii) nicotine volatilized from inhaled particles will be efficiently deposited (Pankow 2001). The effects of tobacco additives on smoke alkalinity is of interest, because as smoke becomes more alkaline, the free-base portion of nicotine (Nic) increases relative to the protonated conjugate acid ($NicH^+$), enhancing the overall nicotine volatility from tobacco smoke particles, and thus facilitating volatilization and transport of nicotine from inhaled smoke particles to respiratory tract surfaces (Pankow 2001). Thus, a shift in the total nicotine to a larger percentage of the free-base assists in making F^{nicotine} greater than $F^{\text{particles}}$.

2 Tobacco Industry Findings

The tobacco industry has extensively researched how product design features, chemical composition, and other qualities of cigarette smoke alter user satisfaction, "kick" or impact (impact is a subjective industry term generally related to a smoker's positive associations with smoke "strength"), and sales. Many reports on this research, summary documents, and internal memos have been made available through disclosure required from legal action taken against the companies. While one must approach these documents somewhat skeptically as they were never intended for public scrutiny and may include speculation and preliminary results, there are consistent themes addressed repeatedly by expert researchers from different tobacco companies. These documents provide insight into various factors that the tobacco industry scientists considered critical for influencing tobacco users to continue using a product that is often harsh and irritating, offensive to nearby nonusers, and leads to death in many of those who use the product. A popular and recurring theme relates interest in smoke pH, clearly showing that tobacco industry scientists spent decades researching and optimizing the influence that additives and physical properties have on the resulting free-base nicotine levels in tobacco-based nicotine delivery products.

2.1 The Concept and Measurement of "Smoke pH"

The term "smoke pH" reflects more of a conceptual idea because the true definition of pH does not necessarily translate clearly to smoke. While many methods used for measuring pH do not determine an accurate value for the smoke's pH and likely provide a biased approximation, broad agreement exists that such measurements often

yield a practical scale by which product comparisons can be made (Chen 1976). These approximations can discern relative differences in smoke pH between tobacco products such as cigarettes (Ingebrethsen et al. 1991) and have been used to compare cigarettes to examine consumer acceptability criteria between brands (Teague 1973). The smoke pH differences between brands are not random occurrences, but are "deliberate and controlled" (Teague 1973) because manufacturers have identified desirable properties in cigarettes with higher pH. Tobacco industry scientists have suggested that the smoke pH from a burning cigarette is one of the key factors altering cigarette smoke properties because it changes the smoke's chemical composition (Chen 1976), including the relative levels of acidic and basic compounds and the equilibrium between conjugate acid–base pairs, including nicotine (Chen 1976). The relation between pH and nicotine's conjugate acid–base equilibrium has been understood by tobacco company researchers for many years (Gregory 1980) and they considered it sufficiently critical to perform numerous research projects to assist in understanding this relationship.

2.2 Free Nicotine and "Smoke pH"

Available tobacco industry documents contain numerous references to free-base nicotine and many of these documents clearly reflect the tobacco industries' evolution of knowledge in this area. For example, industry documents discuss the importance of pH and its influence on the distribution of nicotine between its conjugate acid–base forms. Examples from numerous documents are summarized below to illustrate how the tobacco industry's knowledge of free-base nicotine evolved and how such information was put to practical use.

In one summary document, the tobacco industry characterized cigarettes as an attractive, useful means of delivering nicotine and that a primary concern in developing tobacco products was to maximize both the total nicotine dose and nicotine delivery rate to the user (Teague 1973). The tobacco companies realized that in conjunction with an individual's taste preferences, tobacco use also satisfies a physiological need (Minnemeyer 1976). They indicated that this physiological need is satiated by nicotine, resulting in the addictive properties of the smoke (Minnemeyer 1976). The acid–base chemistry of nicotine was well understood by industry scientists by the 1970s as was the fact that nicotine's free-base form was more irritating or harsher than the protonated form, but transferred across biological membranes more quickly and, thus, was more rapidly absorbed by the smoker (Teague 1973; Reininghaus 1994). Documents referring to smoke pH discussed how tobacco smoke below a pH of 6 contained nicotine exclusively in the nonvolatile, slowly-absorbed singly protonated form, while at higher pH an increasing portion of the nicotine converts to the free-base form, which is volatile, rapidly absorbed, and perceived when smoked as a nicotine "kick" (Teague 1973; Mosser 1984). Numerous tobacco industry executives and scientists were aware how pH influenced the proportion of nicotine existing in the free-base form and its associated

positive smoking attributes (Teague 1973; Schori 1979; Maynor and Rosene 1981; Anonymous 1994; Mosser 1984; Backhurst 1965). Multiple names for free-base nicotine are used in tobacco industry documents (free nicotine, free-base nicotine, vapor phase nicotine, extractable nicotine). These names are often related to how measurements were made and quantitative levels often differ for this reason. However, the concepts are the same, as are the overall qualitative results, and often the terms are used interchangeably (Creighton 1988).

Industry documents discuss how manufactures sought to manipulate the smoke pH, to change the "apparent" nicotine content without altering the absolute yield of nicotine (Chen 1976). This provided an opportunity for the tobacco industry to produce a low "delivery" cigarette, as measured using the currently accepted analytical methods, that continued to provide the user with enough free-base nicotine to meet the needed physiological intake. As they concluded that cigarettes can be designed to deliver significantly more free-base nicotine in smoke (Maynor and Rosene 1981), cigarette manufacturers proposed developing a "low tar" delivery cigarette with relatively high delivery of free-base nicotine (Gregory 1980). As a result of these efforts, they introduced some products on the market that delivered a higher percentage of the total nicotine in the free-base form (Gregory 1980). Tobacco industry scientists recognized that a target amount of free-base nicotine was more desirable than a large amount of bound nicotine (Minnemeyer 1976; Larson and Morgan 1976).

2.3 Use of Ammonia Technology

Both physical design changes to the cigarette and chemical additives to the filler or filter can alter the smoke pH as measured by investigators and, therefore, the relative levels of free-base nicotine (Chen 1976). Competitor tobacco industry scientists suggested that Philip Morris added ammonia and phosphate in the form of diammonium phosphate (referred to as ROOT technology) to adjust the pH of smoke in their products (Gordon 1992; Piehl 1973; Anonymous 1980). In trying to understand the influence of ammonia in cigarettes, an internal RJ Reynolds memo indicated that the use of ammoniated sheet (reconstituted tobacco sheet to which ammonia has been added) by Philip Morris began in 1965 and increased until 1974 (Anonymous 1985). Philip Morris products with the ammoniated sheet added to the filler showed significant improvement, which they described as higher smoke pH, cleaner taste, reduced smoke harshness, and stronger physiological impact (Anonymous 1985). Scientists at RJ Reynolds also investigated the means of increasing the smoke pH by adding basic compounds to the tobacco or to the filter tow (Wilson 1970). These scientists indicated that they had successfully increased their product's smoke pH levels by using mono- and diammonium phosphate and mono- and trisodium phosphate (Wilson 1970). Use of ammonia and/or phosphate was interpreted by Gordon (1992) and Crellin (1985) as enhancing nicotine bioavailability by increasing the delivery of free-base nicotine in the smoke. In some instances, tobacco industry scientists

reported that urea addition to cigarette filler was a source of smoke ammonia (Gordon 1992; Mosser 1984). As urea is pyrolyzed, it forms ammonia and achieves many similar effects as adding ammonia directly.

2.4 Scavenging

While their current practices are unknown, industry documents indicate that Philip Morris used bandcast reconstituted tobacco (a blend of tobacco byproducts formed from a slurry into paper using a casting process) as the primary vehicle for ROOT technology in the 1960s and 1970s (Gordon 1992). Experiments carried out by British American Tobacco scientists suggested that Philip Morris reconstituted bandcast tobacco efficiently and rapidly scavenges nicotine from other tobacco fillers in the immediate proximity (Crellin 1985). Their experiments indicated that as much as 40% of the nicotine in the tobacco lamina can migrate to bandcast reconstituted tobacco. Because bandcast reconstituted tobacco contains diammonium phosphate, Crellin interpreted such results to indicate that nicotine scavenging by bandcast reconstituted tobacco increases free nicotine in the particulate phase (Crellin 1985). Thus, Crellin surmised that a cigarette that was made up of 20% bandcast can deliver 60% more free-base nicotine than if none were present.

2.5 Use of Other Design Features to Alter pH and Free Nicotine

Besides adding ammonia to tobacco filler to alter levels of free nicotine, researchers within the tobacco industry have interpreted their results to indicate that other cigarette design features, including the choice of tobacco leaf, filter design, addition of other basic compounds to the filler, and selective filtration, can also have an effect (Teague 1973; Chen 1976; Backhurst 1965) on free-base nicotine deliveries. They concluded that such results demonstrated that smoke from burley tobacco has higher smoke pH and contains a higher percentage of free-base nicotine than smoke from bright, flue-cured tobacco (Watson 1991; Gregory 1980). Tobacco industry scientists have also reported that the tobacco leaf's stalk position influences nicotine content, pH, and thus the free-base nicotine level in smoke from cigarettes (Creighton 1988). Nicotine concentrations in tobacco stems are low compared to the lamina, but with stems a higher proportion of the nicotine is transferred as free-base nicotine (Crellin 1985). Thus, by altering the tobacco blend, tobacco scientists concluded that both the absolute levels of nicotine and the relative levels of free-base nicotine can be optimized.

Tobacco industry scientists studied filtration technology and indicated that their results showed that this too can have a major influence on smoke pH and free-base nicotine (Chen 1976). They presented results indicating that carbon in filters can increase the pH of the smoke (Creighton 1988). By increasing air dilution of

mainstream smoke, tobacco industry scientists measured higher apparent pH, thus increasing the fraction of nicotine available as the free-base form (Teague 1973). They demonstrated this finding by measuring higher apparent smoke pH values as the filter ventilation increases for the same brand family of cigarettes (Lin and Honeycutt 1984). Results of other studies were interpreted as showing that free-base nicotine levels can be raised by increasing the proportion of burley, reducing casing sugars, using ammonia or other alkaline additives, designing special filter systems that remove acid compounds, or diluting the smoke through filter ventilation or paper permeability (Teague 1973; Anonymous 1980; Schori 1979). Thus, a wide array of tools is at the disposal of the tobacco industry for altering free-base nicotine delivered to the smoker.

2.6 Influence of Free Nicotine on Blood Levels

In trying to understand the influence of free-base nicotine, tobacco industry scientists suggest that free-base nicotine is readily absorbed through the smoker's mucosal and bronchial alveolar lining (Backhurst 1965; Creighton 1988; Reininghaus 1994), rapidly entering the blood stream. They suggested that changes to tobacco smoke free-base nicotine levels affect the nicotine blood levels arising from smoking the product. Because nicotine enters the blood stream more rapidly at higher free-base levels (Creighton 1988), they surmise that higher peak nicotine blood concentrations are achieved (Anonymous 1994) even if the total amount of nicotine entering the blood is not changed. They concluded from these studies that differences in the pharmacokinetics, reflected in nicotine's entry rate to the bloodstream, are an important factor in the smoker's pharmacological satisfaction with a product and the degree to which a particular tobacco product meets the smoker's requirements (Creighton 1988). While independent verification of the relationship between blood levels and smoker satisfaction for cigarettes has not been performed, data on smokeless tobacco has shown a clear relationship between blood levels and free nicotine in these products (Fant et al. 1999).

2.7 Influence of pH and Free Nicotine on Taste, Harshness, Impact and Sales

Impact has been loosely defined by some tobacco industry scientists as the degree of awareness of the presence of tobacco smoke in the back of the smoker's throat (Schori 1979), or as an involuntary reflex related to the pharmacology of nicotine (Maynor and Rosene 1981). Impact primarily results from physiological changes taking place upon exposure to nicotine. But, it is more a complex phenomenon than just simple exposure to total nicotine, evidenced by findings in which cigarettes with identical total nicotine deliveries have very different impacts (Gullotta et al. 1990).

Experiments carried out by the tobacco industry concluded that cigarettes containing equimolar amounts of nicotine, but which differ in their basic or acidic properties, produce different subjective and electrophysiological effects in smokers (Gullotta et al. 1990; Anonymous 1994; Backhurst 1965), with cigarettes having higher free-base nicotine being considered "stronger." Their experimental results showed that as the alkalinity of mainstream tobacco smoke increases, greater physiological impact occurs in the throat and chest (Chen 1976; Ingebrethsen et al. 1991), resulting in elevated electrophysiologic and subjective responses (Chen 1976; Anonymous 1994). Tobacco industry scientists interpreted these findings to suggest that, because free-base nicotine is a more physiologically effective form (Anonymous 1994), increasing alkalinity causes nicotine to be more "available" to the smoker, improving nicotine satisfaction (Schori 1979).

There was a concerted effort within research and development at RJ Reynolds, Philip Morris, and Brown and Williamson to modify cigarette smoke properties to deliver the most physiologically effective form of nicotine for obtaining the greatest impact or kick, while maintaining acceptability. Because they determined that greater impact occurs with smoke from burley tobacco (Gregory 1980), cigarette tobacco blends were carefully researched. Their results showed that the pH of American blend filler is about 5.5, but that the smoke pH is higher (Anonymous 1994). When the tobacco filler pH is approximately 6.4, tobacco industry studies showed that a stronger physiological effect is perceived by the smoker, resulting directly from an increase in the amount of free-base nicotine in smoke at the higher pH (Anonymous 1994). When competing companies examined cigarettes from the market leader Marlboro, they reported that Marlboro smoke had higher pH and contained more free-base nicotine, providing the smoker with a more instantaneous nicotine kick (Teague 1973).

A trade-off exists when raising the alkalinity and, consequently, the amount of free-base nicotine in the smoke. At higher pH levels, smoke becomes harsh and unpleasant (Chen 1976). While smokers sense an increased impact associated with smoke pH, there is also increased irritation if the alkalinity is raised too far (Mosser 1984). Tobacco industry studies have suggested that at a smoke pH above 7, the smoke becomes perceptibly harsh and a break-over point exists, yielding a smoke that is too harsh and thus undesirable to the smoker (Teague 1973). To achieve a cigarette smoke that is acceptable to consumers, manufacturers balance the undesirable harshness of smoke with the preferred level of physiological fulfillment (Chen 1976; Mosser 1984). Thus, one objective in cigarette design is tailoring smoke chemistry to achieve a "smooth" smoke that rapidly delivers nicotine in a pleasing manner. Tobacco industry documents discuss the major changes that occurred in the design of Marlboro cigarettes, starting in the 1960s (Dickerson 1977). Additional changes, focused on smoke pH, were made yearly in Marlboro cigarettes in the early 1970s (Dickerson 1977). These documents discuss systematic increases in smoke pH, resulting in smoke pH values consistently higher than other competitive high selling brands, such as Winston, which the scientists interpreted to indicate a desired increase in free-base nicotine delivery (Dickerson 1977). In subsequent

years, documents suggest that Philip Morris had to reduce the pH because it became too high (Dickerson 1977), making the smoke harsh and less acceptable.

Cigarette market leaders learned how to harness free-base nicotine to provide the kick and physiological impact that smokers desire without the product being too harsh (Teague 1973; Chen 1976). Tobacco industry research and development documents have suggested that smoke pH has a direct effect on nicotine impact and hence on market performance (Teague 1973; Anonymous 1980; Christopher 1973). They have reported that as much as 50% of the variation in sales could be a consequence of smoke pH (Anonymous 1980). A 1973 report indicated that free-base nicotine was the highest determinant for sales between the four leading brands (Blevins 1973). By examining the relationship between design changes and sales, industry documents indicate that the use of ammoniated sheet by Philip Morris corresponded to a dramatic increase in Philip Morris sales in the early 1970s (Anonymous 1985) and that the historical sales and market performance of Marlboro correlated closely with smoke pH and free-base nicotine levels (Teague 1973; Piehl 1973; Anonymous 1980). RJ Reynolds evaluated the properties of Marlboro and Kool cigarettes to determine why their sales increased so quickly (Teague 1973). They found that Marlboro cigarettes contained three times more free-base nicotine than their own flagship Winston brand and concluded that the difference in sales was directly related to an increase in free-base nicotine as a result of adjustments in smoke pH (Teague 1973). An improvement in RJ Reynolds' market occurred in 1974 with the introduction of ammoniated sheet technology (Anonymous 1985).

The documents released by tobacco companies as a result of settlements of legal action provide a stimulating picture of their efforts to modify tobacco products to increase sales of a product that is inherently deadly. By adjusting free-base nicotine levels to achieve a balance between nicotine impact and harshness through tobacco blending, additives, filter ventilation, selective filtration, and other modifications, they are able to provide a smoke that has more impact and rapidly delivers nicotine to the blood stream. Clearly, successful implementation of such technology is a major determinant of smoker satisfaction and is an important determinant of increased sales and continued use of the product. In addressing cessation, initiation, and reduction in overall harm from these products, the influence of these and future design changes must be addressed.

3 Free Nicotine in Tobacco Smoke

As described above and by Wayne et al. (2006), numerous documents within the Legacy Tobacco Documents Library (http://legacy.library.ucsf.edu/) demonstrate the decades-long interest that tobacco industry scientists have had in measuring the fraction of nicotine that is in the free-base form, which has been denoted α_{fb} by Liang and Pankow (1996). Mainstream smoke is a dynamic and continuously evolving stream of aerosol particles. As smoke exits the cigarette, physical and chemical changes in the smoke stream occur at a rapid rate (Borgerding and Klus 2005).

Volatile species evaporate from individual aerosol droplets and the droplets begin to rapidly coalesce together forming droplets with larger radii. Ambient humidity may influence evaporation, and the rate of aerosol particle coalescence is a function of time and particle density that varies with filter type, filter ventilation, paper porosity, tobacco filler packing density, and filler cut-width. For these reasons and those described by Pankow (2001), none of the previous measurements (including those by the "smoke pH" method) were capable of leading to reliable estimates of α_{fb} . It was not until the appearance of the study by Pankow et al. (2003) that a dependable and accurate method existed for measuring α_{fb} values. Watson et al. (2004) describe similar results by a different method. It should be noted that scientists connected to the tobacco industry dispute that α_{fb} values for tobacco smoke collected from cigarettes are of importance in affecting the efficiency of step 2, the delivery of nicotine from the smoke to the smoker. For example, citing results of work conducted when he was employed with RJ Reynolds, Ingebrethsen (2006) asserted that the high observed deposition efficiency values for nicotine, F^{nicotine}, for unspecified commercial cigarettes argue for the relative unimportance of α_{fb} rather than for its importance.

Nicotine α_{fb} values in tobacco smoke particulate matter can be estimated based on nicotine volatility from the smoke particulate matter phase, as controlled by the gas/partitioning constant K_p (Pankow et al. 1997):

$$K_p(\text{m}^3\mu\text{g}^{-1}) = \frac{c_p}{c_g}. \tag{3}$$

Here c_p (ng μg^{-1}) is the total (protonated + free − base) nicotine concentration in the particulate matter phase and c_g (ng m^{-3}) is the equilibrium concentration of nicotine's free-base form in the gas phase. Only the free-base form has appreciable volatility and would be present in the gas phase, so only the free-base form of nicotine can transfer between the particle and gas phases. The concentration of free-base nicotine is $\alpha_{fb}c_p$. An underlying partitioning constant for free-base nicotine is given by (Pankow et al. 1997; Pankow 2001):

$$K_{p,fb} = K_p\alpha_{fb} \tag{4}$$

or

$$\alpha_{fb} = \frac{K_{p,fb}}{K_p}. \tag{5}$$

Knowledge of K_p and $K_{p,fb}$ gives α_{fb} for particulate matter. Brand-dependent nicotine α_{fb} values at 20°C for mainstream smoke particulate matter after collection can be determined by measuring the initial gas concentration $c_{g,1}$ in a smoke sample. A base such as gaseous ammonia can be introduced to the collected smoke to convert the remaining nicotine to the free-base form and generate the equilibrium concentration $c_{g,2}$. The percentage of free-base nicotine is computed by determining α_{fb} where:

$$\alpha_{fb} = c_{g,1}/c_{g,2}. \tag{6}$$

Multiple publications (Pankow et al. 1997; Ingebrethsen et al. 2001; Pankow et al. 2003; Watson et al. 2004) have discussed measuring free-base nicotine directly, addressed the importance of free-base nicotine delivery, and examined the chemical properties of nicotine in cigarette smoke as an important determinant of the effective delivery and bioavailability of nicotine from cigarettes. Pankow et al. (1997) examined how ammonia influences nicotine delivery in tobacco smoke and concluded that conversion of nicotine to the free-base form could be facilitated by ammonia. Based on a theoretical treatment, Pankow et al. (1997) concluded that, under certain circumstances, up to 40% of the nicotine could be available as the volatile free-base form. These authors also concluded that the rate of volatilization was more rapid than that previously measured by Lewis et al. (1995) using denuder technology to examine the properties of mainstream cigarette smoke.

A continuation of the denuder work by Ingebrethsen et al. (2001) found that about 10% of the total nicotine in domestic cigarettes volatilizes rapidly and could be readily absorbed. In addition, they confirmed that burley tobacco, because of its native higher alkalinity, has a greater fraction of nicotine available as the free-base form than in bright tobacco. Their findings that about 10% of nicotine exists as free-base nicotine are consistent with the average of 13% reported by Watson et al. (2004) from headspace measurements above mainstream cigarette smoke particulate matter generated by standardized machine smoking from 26 cigarette brands.

Pankow et al. (2003) published the first report on directly measuring the levels of free-base nicotine in whole mainstream smoke from commercial cigarettes. Whole mainstream cigarette smoke collected in air sampling bags was sampled by pulling a known volume through a Teflon membrane filter with subsequent collection on glass sampling cartridges containing approximately 0.1 g of Tenax-TA sorbent. Cartridges were analyzed by thermal desorption and gas chromatography mass spectrometry (GC/MS) using nicotine-D_3 as an internal standard. The ability of Tenax-TA cartridges to quantitatively collect and release gas phase nicotine was verified. After anhydrous gaseous ammonia was added to the remaining smoke in the collection bag, $c_{g,2}$ values were determined in a similar manner. The c_p value for nicotine in each bag was determined by weighing the exhausted bag to determine the particulate matter's mass, extracting with low-water isopropanol, and determining the nicotine by GC/MS. Values of $\alpha_{fb} = c_{g,1}/c_{g,2}$ and $K_p = c_p/c_{g,1}$ were calculated for each smoking event. This publication showed that, not only was most nicotine associated with the particulate matter, but that the latter contained both protonated and free-base nicotine. Determination of the ratio of free-base to protonated nicotine (α_{fb}) was made on the first three puffs and then on the remaining puffs. Based on the analysis of 11 commercial brands and a standard research cigarette (University of Kentucky 1R4F), seven of the brands had higher α_{fb} in the first three puffs than in the remaining puffs, two brands had the same α_{fb} in the early and later puffs, and three brands had lower α_{fb} in the first three puffs compared to the remaining puffs. Although their data suggested a trend of relatively higher free-base nicotine in the initial puffs for the majority of the commercial brands, the confidence intervals were sufficiently wide to limit the ability to draw final conclusions.

Watson et al. (2004) measured headspace nicotine above total particulate matter collected on a Cambridge filter pad (CFP) by solid phase microextraction (Zhang and Pawliszyn 1993) with GC/MS detection using deuterated toluene as an internal standard. All measured nicotine in the headspace was attributed to the free-base form, since the protonated form has substantially lower volatility. Free-base nicotine in smoke might be expected to remain in the vapor phase portion of mainstream smoke and pass through the CFP. To investigate this possibility, they examined the gaseous materials that passed through the CFP (normally referred to as the vapor phase portion of mainstream smoke). In agreement with Pankow et al. (2003), no significant amount of nicotine was detected in the vapor phase portion of tobacco smoke, although other smoke constituents were readily detected at the nanogram to milligram levels. The most likely reason that free-base nicotine remains associated with the relatively wet particulate phase is nicotine's high water solubility. These authors also found that cigarettes with higher ventilation had, on average, higher percentages of nicotine in the free-base form. The average percentage of free-base nicotine for full-flavored ($n = 11$), light ($n = 9$), and ultra light ($n = 6$) cigarette brands was 7, 11, and 28%, respectively. Surprisingly, the free-base nicotine had only weak correlations with tobacco filler pH or ammonia content, but showed a significant relation to filter ventilation levels. Mainstream smoke from cigarette brands with higher levels of filter ventilation is highly diluted with air, minimizing coalescence of the aerosol particles and providing additional off-gassing opportunities from the particles to the vapor phase.

Although the Pankow et al. (2003) method, which analyzed whole mainstream smoke captured in a gas sampling bag, and the Watson et al. (2004) method, which performed headspace analysis of total particulate matter collected on a standard glass–fiber filter, employed different analytical approaches, excellent agreement was found for free nicotine levels for the same brand variants. Pankow et al. (2003) measured the effective pH of the first three and last three puffs, while Watson et al. (2004) measured all puffs. The two approaches showed excellent agreement in α_{fb} between the two methods for all seven overlapping brand variants. The ability to use different techniques, in different laboratories, helps illustrate the validity for measuring the form of nicotine delivered to the smoker. It could be the case that a so-called low-delivery light or ultra-light style cigarette with a high percentage of nicotine available as the free-base form could be equally or more addictive than a high-delivery full-flavored cigarette having relatively low free-base nicotine levels. Also, cigarette brands with differing levels of free-base nicotine could alter a smoker's normal smoking pattern, resulting in differing exposure to the other toxic chemicals in tobacco smoke.

Virtually all cigarettes deliver enough nicotine to sustain addiction and are designed so that smokers are able to adjust their smoking habits to meet their personal preferences and physiological needs. Due to the way that smokers interact with the product (taking puffs of varying size, puffing at different rates, blocking or leaving ventilation holes open) smokers can alter the amount of total nicotine received and the fraction that is in the free-base form. Because cigarettes are an elastic product, smokers can adjust their smoking behavior to modify delivery. This is in

contrast to smokeless tobacco, for which the product characteristics are the primary determinant of nicotine absorption for a given dose (Fant et al. 1999; Henningfield et al. 1995).

4 Free Nicotine in Smokeless Tobacco

Although scientists have used multiple approaches in attempting to characterize pH and free-base nicotine in mainstream smoke, more straightforward and consistent approaches have been used to measure pH, total nicotine, and calculated free-base nicotine content in smokeless tobacco products (CDC 1999a, b; Richter and Spierto 2003; Djordjevic et al. 1995; Henningfield et al. 1995; Ayo-Yusuf et al. 2004). Generally, a fixed volume of distilled water (previously boiled or sparged to remove dissolved carbon dioxide) is added to a known amount of the smokeless tobacco product and the resulting pH of the mixture is determined by a continuous series of measurement over 30 min. Nicotine content and pH are used with the Henderson–Hasselbalch equation (CDC 1999a) to calculate the fraction of nicotine in the free-base form. Because of tobacco's buffering capacity, the derived result is not substantially influenced by the amount of water used to suspend the tobacco. This approach is simpler and easier to measure than methods used for tobacco smoke, and the free-base nicotine data from smokeless tobacco products is less ambiguous than that from tobacco smoke.

Significant variations in the reported free-base nicotine levels in smokeless tobacco products illustrate that considerable differences exist in this category of products. Even though pH, moisture, total and free-base nicotine levels are reported annually to the Department of Health and Human Services by the tobacco companies, by law this information cannot be made public in accordance with 5 U.S.C. 552(b)(4) and 18 U.S.C. 1905. Separate from this reporting requirement, independent investigations have made measurements on a range of smokeless products, primarily moist snuff available in the United States. In 1995, Djordjevic and coworkers (Djordjevic et al. 1995) examined the levels of moisture, pH, total nicotine, and free-base nicotine in 17 moist snuff brands (Table 1). They measured the pH of these products and reported a range of 5.39–7.99. These values yielded percent free-base nicotine ranging from 0.23 to 48.3%, reflecting the dramatic increase in free-base nicotine obtained over this pH range. Total nicotine also varied significantly from 3.4 to 14.5 mg g^{-1}, showing clear brand-specific differences. A wide range of pH and total nicotine determined in these samples contributed to a corresponding wide range of free-base nicotine levels (Table 1). Also in 1995, Henningfield et al. (1995) examined the nicotine content and pH of six different moist snuff tobacco products, including some of the highest sellers from three different regions of the United States. They reported total nicotine concentrations ranging from 7.5 to 11.4 mg g^{-1} and pH from 6.9 to 8.6. The measured pH values corresponded to the percentage of free-base nicotine in these products, ranging from 7 to 79%. A similar study was carried out by the Centers for Disease Control and

Table 1 Values of pH and levels of free nicotine in smokeless tobacco products

Year	Products	pH	Free nicotine (%)	Free nicotine (mg g^{-1})	Reference
1995	17 brands of moist snuff	5.39–7.99	0.23–48.3	0.028–5.6	Djordjevic et al. (1995)
1995	Six brands of moist snuff	6.9–8.6	7.05–79.2	0.53–9.0	Henningfield et al. 1995
1999	Six brands of moist snuff	5.24–8.35	0.23–68.1	0.01–6.23	CDC, 1999b
2003	Eight brands of moist snuff	5.35[a]–8.28	0.20[a]–64.5	0.01[a]–5.81	Richter and Spierto (2003)
2003	Ten brands of loose leaf	5.33–6.41	0.20–2.44	0.02–0.11	Richter and Spierto (2003)
2004	Traditional South African product	8.2	60.2	9.63	Ayo-Yusuf et al. (2004)
2004	Commercial South African smokeless tobacco	7.1[b]–10.1	10.1[b]–99.2	1.6[b]–12.9	Ayo-Yusuf et al. (2004)
2005	Iqmik	10.2	–	–	Renner et al. (2005)

[a]Except for one brand of moist snuff, lowest pH would have been 7.13, lowest % free nicotine would have been 11.4, and lowest level of free nicotine would have been 0.97

[b]Except for one brand, lowest pH would have been 8.8, lowest % free nicotine would have been 86.1%, and lowest level of free nicotine would have been 5.95 mg g^{-1}

Prevention (CDC) and the Department of Health in Florida, which looked at six moist snuff products (CDC 1999b). The six moist snuff products from six cities in Florida were analyzed for pH, nicotine content, and moisture and the free-base nicotine levels derived from these measurements varied from 0.01 to 6.23 mg g^{-1}, corresponding to the 0.23–68.14% nicotine in the free-base form. More recently, Richter and Spierto (2003) examined levels of nicotine, pH, moisture, and free-base nicotine in 18 brands of moist snuff and loose-leaf smokeless tobacco. This study differed from the others by including loose-leaf smokeless tobacco in addition to moist snuff. Nicotine content in the moist snuff products was 4.28–13.54 mg g^{-1}, somewhat higher than the 3.73–8.26 mg g^{-1} found in loose-leaf products (Table 1). Overall, measurements on moist snuff products in different laboratories are generally in good agreement and indicate that the range of free-base nicotine levels spans orders of magnitude, with the percentage of nicotine in the free-base form in some cases exceeding 50%. Thus, for moist snuff products, users can be exposed to very different free-base nicotine levels depending on the product used. In loose-leaf tobacco products, the lower tobacco pH influenced the free-base nicotine levels so that they were much lower than most of the moist snuff products.

In addition to analysis of smokeless products commonly used in the United States, a few studies have examined free-base nicotine levels in tobacco products

from other countries. A traditional South African smokeless tobacco product and other commercial tobacco products were tested using the same methods as performed in the analysis of US moist snuff above (Ayo-Yusuf et al. 2004). Except for one commercial product, all pH values, percent free-base nicotine, and levels of total nicotine were in the upper range or higher than moist snuff samples from the United States (Table 1). In some cases, the pH was sufficiently high that virtually all nicotine from these samples was in the free-base form. A single measurement was reported from the analysis of the pH of iqmik, a smokeless tobacco product consumed in Western Alaska and produced by prechewing a mixture of leaf tobacco and an alkaline ash from punk fungus (Renner et al. 2005). The pH value of 10.2 reported for this product is sufficiently high that it corresponds to a product preparation in which virtually all the nicotine is available as the free-base form. In India, smokeless tobacco products take on many different forms. Analyses of Indian smokeless tobacco nicotine and pH levels suggest that products such as gutkha and khaini, which are popular in India, also have high pH values and free nicotine content (Gupta PC, personal communication). Thus, the levels of free nicotine in smokeless tobacco may be an even larger issue than is reflected by analysis of commercial US moist snuff or loose-leaf chewing tobacco.

Nicotine's delivery rate from smokeless tobacco products is a function of the total nicotine level, product pH, various additives that alter the form of nicotine or increase its availability, and the tobacco cut size (Henningfield et al. 1995, 1997). Variation in free-base nicotine levels in different smokeless tobacco products combined with user questionnaire data suggest that a series of brands, designated as starter brands, are developed specifically for the novice user (Fant et al. 1999; Henningfield et al. 1995, 1997; Djordjevic et al. 1995; Tomar et al. 1995). In these products, free-base nicotine levels are sufficiently low that noxious properties associated with nicotine exposure are minimized for the novice users. In order to avoid heart pounding, vomiting, and other symptoms associated with acute nicotine toxicity (Henningfield et al. 1995; Tomar and Henningfield 1997), new users must absorb less nicotine than the maximum biologically available dosages in most products. Therefore, low-nicotine delivery products are available in a more palatable variety for beginning users. As tolerance develops, the low-nicotine delivery products no longer meet the needs of a seasoned user (Tomar and Henningfield 1997), who now requires tobacco products with increased nicotine delivery (Henningfield et al. 1997). As users require additional nicotine doses to achieve the desired physiological impact and as they become accustomed to the pharmacological effects of nicotine, they switch to different tobacco products that deliver higher levels of biologically available nicotine. Different smokeless tobacco products are manufactured with nicotine deliveries, both in terms of total and free-base nicotine levels, spanning a wide range to yield a graduated tier of available nicotine delivery products. This range of free-base nicotine ensures that sufficient diverse delivery products are available to satisfy the requirements of the novice through to the seasoned smokeless tobacco user.

5 Summary

Evidence from the tobacco industry documents, from research studies that measure free-base directly in tobacco smoke particulate, and from examination of smokeless tobacco products, all show that the level of free-base nicotine as delivered to the tobacco user is a critical variable in the acceptance of tobacco products and their continued use. The physiological impact of the rapid delivery of nicotine in the free-base form is a critical determinant of continued nicotine-seeking behavior, with the unintended consequences of exposure to the other toxic components of tobacco smoke and smokeless tobacco. Evaluating total delivered nicotine alone is not sufficient to characterize product differences. To fully understand the influence nicotine has on the allure of these products, both total and free-nicotine levels must be measured. A comprehensive understanding of nicotine delivery is needed to help find effective means for breaking its addictive nature and, ultimately, in reducing the morbidity and mortality associated with tobacco use. The levels of free-base nicotine must be included as part of any effort to achieve a better understanding of how tobacco products themselves influence their continued use.

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Tobacco Industry Manipulation of Nicotine Dosing

Geoffrey Ferris Wayne and Carrie M. Carpenter

Contents

Abstract For more than a half century, tobacco manufacturers have conducted sophisticated internal research to evaluate nicotine delivery, and modified their products to ensure availability of nicotine to smokers and to optimize its effects. Tobacco has proven to be a particularly effective vehicle for nicotine, enabling manipulation of smoke chemistry and of mechanisms of delivery, and providing sensory cues that critically inform patterns of smoking behavior as well as reinforce the impact of nicotine. A range of physical and chemical product design changes provide precise control over the quantity, form, and perception of nicotine dose, and support compensatory behavior, which is driven by the smoker's addiction to nicotine. Cigarette

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manufacturers also enhance the physiological effects of nicotine through the introduction and use of compounds that interact with nicotine but do not directly alter its form or delivery. A review of internal documents indicates important historical differences, as well as significant differences between commercial brands, underscoring the effectiveness of methods adopted by manufacturers to control nicotine dosing and target the needs of specific populations of smokers through commercial product development. Although the focus of the current review is on the manipulation of nicotine dosing characteristics, the evidence indicates that product design facilitates tobacco addiction through diverse addiction-potentiating mechanisms.

1 Introduction

Tobacco manufacturers have long been aware that nicotine is the central component of tobacco dependence, and that sales, and ultimately profits, depend on creating and sustaining that dependence (Anderson and Read 1980; Teague 1972; Templeton 1984; Yearnan 1963). As observed by a researcher for Philip Morris in 1972: "The cigarette should not be construed as a product but a package. The product is nicotine" (Dunn 1972). It is not surprising then that the available evidence demonstrates a consistent and far-reaching record of internal industry research into the role and function of nicotine, and the physical and chemical product design parameters that influence the delivery of nicotine to smokers.

Examples of manufacturers' concern with the role of nicotine dosing in cigarette smoking appear as early as the 1950s. An internal Philip Morris memorandum describes the use of "informally constituted smoking panels and the results of nicotine analyses performed on the blends… to establish a desirable level of nicotine concentration in the blend and hence also in the smoke" (Philip Morris 1954). Subsequent industry documents highlight the continued significance of internal research for determining optimum quantities and forms of delivered nicotine. Major cigarette manufacturers have sought to optimize the dose and effects of nicotine:

1963 A Brown & Williamson (BW) letter discussed "optimum levels" for nicotine and noted "we have a research program in progress to obtain… any level of nicotine desired" (Griffith 1963).

1978–1984 R.J. Reynolds (RJR) maintained a "nicotine optimization program," the goals of which were to define "the optimum nicotine level in cigarette smoke required to maximize smoker satisfaction" as well as "a minimum or threshold value of nicotine required for satisfaction" (Piehl 1978; R.J. Reynolds 1989).

1980 A Lorillard memo outlined an internal project to "determine the minimum level of nicotine that will allow continued smoking" (Smith 1980).

1984 A British American Tobacco (BAT) conference presentation described a "research program to meet the criteria for maximizing nicotine effects to satisfy consumer needs from a minimum dose of nicotine" (British American Tobacco 1984a).

1990 A mission statement for RJR's Pharmacology Division identified "optimum nicotine" as a priority and outlined an integrated program of research "to define the role of nicotine in smoker satisfaction and optimize nicotine as a product design parameter" (R.J. Reynolds 1990b).

1990 Philip Morris researchers noted as internal achievements the findings "that there are optimal cigarette nicotine deliveries for producing the most favorable physiological and behavioral responses" and "that all forms of nicotine are not behaviorally or physiologically equal" (Gullotta et al. 1990a).

1992 An RJR research plan described a "nicotine dose–satisfaction study," which was "designed to establish the minimum smoke nicotine level required to provide normal plasma nicotine and smoking behavior responses…" (Fluhler 1992).

The goal of these efforts was to ensure that smokers obtained sufficient nicotine to support dependence. Cigarettes were developed and marketed on the premise "that the primary motivation for smoking is to obtain the pharmacological effect of nicotine" (Philip Morris 1969) and consequently, with the understanding that they "must provide the appropriate levels of nicotine" (Brown & Williamson 1977).

Sophisticated internal methods were developed to assess dose and effects. These measures included physical product analyses; machine-based and human-based smoke yields; individual smoking patterns; metabolites; smoker characteristics; and smoker perceptions and response (Philip Morris 1981; R.J. Reynolds 1987b, 1991, 1994). Techniques were drawn from a range of disciplines such as electrophysiology, experimental psychology, and behavioral pharmacology (Laurene 1977; Jeanneret 1975; Philip Morris 1977, 1964; Read 1984; Reininghaus 1987; Piehl 1978; R.J. Reynolds 1989; Griffith 1963). Further product adjustments (e.g., ventilation, additives) were used to optimize the effects of nicotine, enabling cigarette manufacturers to enhance addiction even while maintaining levels of nicotine (Henningfield et al. 2004a).

Internal evidence demonstrates a highly developed understanding of tobacco use and addiction. Further, the evidence confirms the application of this knowledge to commercial product design, and the manipulation of nicotine and other physical and chemical design parameters to ensure availability of nicotine to smokers. As summarized in Fig. 1, industry scientists optimized nicotine dosing, combining behavioral research and product technology to maximize nicotine effects, and, thus, aimed to improve commercial products and maintain smoking and addiction. Manufacturers continuously evaluated consumer response, including individual smoker characteristics (e.g., demographics, personality) and behavior, to inform increasingly sophisticated product development and design-targeted commercial cigarettes.

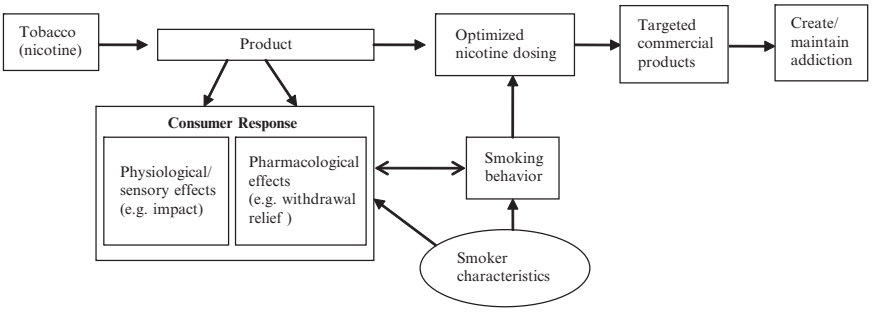

Fig. 1 Industry manipulation of ninotine delivery

2 Factors in Nicotine Dosing

Nicotine is commonly measured in smoke using a smoking machine, which draws smoke from the lit cigarette under a set of standard parameters (volume, puff duration, puff interval). While machine-based methods provide a means for quantifying relative product differences, they are generally recognized to be inadequate, and even deceptive, with respect to their application to smokers (Hammond et al. 2006, 2007; National Cancer Institute 2001). This is primarily due to their inability to account for either behavioral (e.g., compensatory) differences in product use, or differences in the chemical form of nicotine. Increasingly, machine-based analyses are giving way to more sophisticated techniques, many of which are derived from internal industry methodologies (see chapter by Ashley, this volume).

Internal research supports the conclusion that multiple factors interact to determine the effects of tobacco use: sensory and physiological; social and personal; physical and behavioral (see Fig. 2, taken from a 1992 RJR document; Green 1992). It is now recognized that modern cigarettes can be engineered to increase the effectiveness and bioavailability of nicotine (Bates et al. 1999; Pankow 2001; US Food and Drug Administration 1995, 1996); to control sensory effects, including ease of inhalation and trigeminal impact (sensory "bite" or "throat grab") (Ferris Wayne and

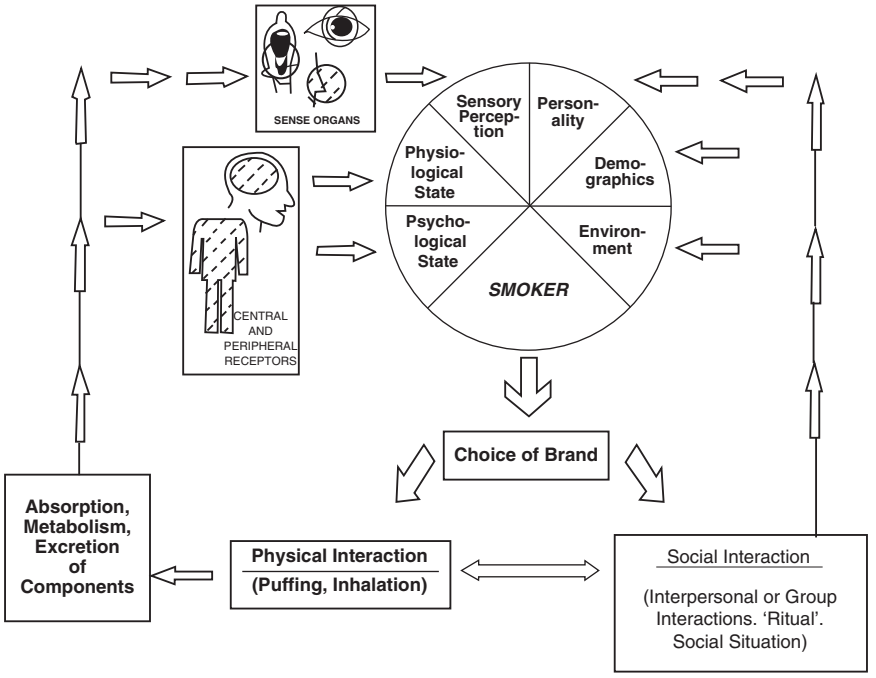

Fig. 2 Illustration of "the determinants of smoking satisfaction" (Green et al. 1992)

Connolly 2004; Pankow 2001; Wayne and Connolly 2002); and to target the needs of specific smoker populations (Carpenter et al. 2005; Cook et al. 2003).

2.1 Smoke Nicotine Yield

Despite noted limitations of machine-based methods, these measures can provide information on commercial trends in smoke nicotine delivery. For example, the US Food and Drug Administration observed a trend of increasing smoke nicotine yield from 1982 to 1991, with the greatest increases in the lowest-tar cigarettes (Kessler 1994). This trend strongly suggested that manufacturers had manipulated and controlled the levels of nicotine (Kessler et al. 1996). Similarly, in a recent analysis of machine-based nicotine yield data provided by manufacturers to the Massachusetts Department of Public Health, a small but statistically significant trend in increased smoke nicotine yield was observed from 1997 to 2005. The increasing trend was observed within all major market categories including full-flavor, light, medium, and ultralight; mentholated and nonmentholated; and within each major manufacturer, though at varying rates (Connolly et al. 2007).

It must be noted that smoke nicotine represents only a small fraction of the nicotine contained within the unburned cigarette and, further, that all commercial cigarettes house sufficient nicotine to sustain dependence (Henningfield et al. 1998). The availability of nicotine from a single cigarette is highly variable and remains dependent on changes in smoking patterns (e.g., larger puff volume, more frequent puffs, vent blocking) (Hammond et al. 2005; Kozlowski and O'Connor 2002). Indeed, compensatory behaviors have been demonstrated to increase the amount of nicotine delivered to as much as eight times the machine-measured yield (Jarvis et al. 2001). It is unknown whether a relatively small increase in machine-measured smoke nicotine yield, such as described above, is likely to reflect a real increase in the available dose. Nonetheless, it provides an indication of the need for further monitoring and assessment to understand industry-wide product changes.

In the above study, Connolly et al. (2007) found that the increase in smoke nicotine yield reflected an underlying increase in nicotine concentration within commercial cigarettes, suggesting a greater ease at obtaining the nicotine dose within a given puff. This trend was further supported by the observed increase in per-puff smoke nicotine yield. Increasing the availability of nicotine in cigarette tobacco means that cigarettes do not need to be smoked as intensively, or fewer cigarettes can be smoked to achieve the same daily level of nicotine intake. These findings illustrate how an apparent increase in smoke nicotine yield could in fact facilitate the ease with which a smoker obtains a given dose of nicotine, while supporting the same total nicotine dose.

2.2 Tobacco as a Vehicle for Nicotine Delivery

While nicotine is the primary active pharmacological agent, tobacco has been shown to be a particularly effective vehicle for delivery of nicotine (US Food and Drug Administration 1995; Hurt and Robertson 1998; Slade et al. 1995; World Health Organization 2001). In fact, published research has determined that tobacco-delivered nicotine is not only more toxic, but more addictive than nicotine in a pure form (e.g., nicotine replacement therapy) (Henningfield et al. 2000; Royal College of Physicians 2000). As noted by a BW scientist in 1990: "Nicotine alone in smoke is not practical, nor are extreme tar/nicotine ratios, since nicotine is too irritating – other substances are required for sensoric reasons" (Baker 1990).

The importance of tobacco includes both those constituents in smoke that may interact with nicotine directly, as well as those that indirectly influence a smoker's perception and behaviors. For example, some tobacco smoke constituents may alter the site of absorption of nicotine, such as bronchodilators (e.g., cocoa, licorice), which allow deeper inhalation and subsequent deposition of constituents in more highly permeable areas of the respiratory tract. Likewise, product changes to alter or control particle size, or to provide particulate "carriers" for vapor-phase smoke constituents, also could facilitate changes at the site of absorption (Ingebrethsen 1993). This would also include the use of acids or bases to alter the form of nicotine and basicity of smoke. Again, a wide range of relevant findings is indicated by internal documents (Ferris Wayne et al. 2006; Keithly et al. 2005; Pankow 2001).

Alternately, flavorants (e.g., menthol) and smoke "smoothing" agents (e.g., sugars) may be used to mask or balance irritation and thereby facilitate nicotine dosing by offsetting the harshness of nicotine and removing natural physiological barriers (Burns 1992; National Cancer Institute 2001; Wayne and Connolly 2002; Wells 1995). Other approaches to reducing harshness would include the addition of a "cooling" or anesthetic compound (e.g., menthol, eugenol), altering smoke composition (such as the T/N ratio), or removing other tobacco constituents with irritant properties (Bates et al. 1999; Ferris Wayne and Connolly 2004; Hurt and Robertson 1998; Pankow 2001; Wayne et al. 2004). Reduced irritation may encourage or support increased frequency of use, and has been linked to increased rates of initiation and uptake of smoking among youth (Wayne and Connolly 2002).

2.3 Chemical Form of Nicotine

In cigarette smoke, nicotine may exist in either its protonated (bound) or unprotonated (free) forms, with most traditionally assumed to be protonated. A greater percentage of nicotine delivered in its unprotonated form, as determined by smoke basicity (often referred to as "smoke pH"), may result in increased rates of absorption in the mouth and upper respiratory tract (increasing sensory impact through stimulation of receptors), as well as faster absorption from the lower respiratory tract to the

brain (Henningfield et al. 2004b; Hurt and Robertson 1998). These changes could alter the physiological response of smokers. Consequently, the chemical form of nicotine delivered by cigarettes has received increasing attention from public health researchers (Hurt and Robertson 1998; Kessler 1994).

Internal industry documents provide overwhelming evidence that manufacturers recognized and exploited differences in the chemical form of nicotine. For example, manufacturers monitored the free nicotine levels of competitor brands in the 1970s and found strong correlations between free nicotine delivery and market share (Leach and Shockley 1969; Teague 1973; Woods and Sheets 1975). Further, manufacturers identified free nicotine as a means to increase physiological satisfaction in lower nicotine products (Gregory 1980; Larson and Morgan 1976; Lorillard 1973; Schori 1979). As concluded by Philip Morris researchers in 1990, a low nicotine delivery cigarette with a higher proportion of free nicotine "would be analytically similar to other cigarettes at comparable nicotine deliveries, but would be judged to have much more impact" (Gullotta et al. 1990c). Thus, manipulation of the form of nicotine was seen both as a means to maintain a competitive advantage across brand styles, and as a means to replace physiological impact among low nicotine cigarettes (Ferris Wayne et al. 2006; Hurt and Robertson 1998; Pankow 2001).

Pankow (2001) evaluated the considerable research conducted by tobacco manufacturers to describe patterns of deposition of nicotine related to differences in acid/base chemistry of smoke, noting the likely importance of "smoke pH" and unprotonated (free) nicotine in smoke delivery. This research led to independent confirmation of the utility of analytic techniques for assessment of the form of nicotine, demonstrating significant brand differences in free nicotine delivery (Pankow et al. 2003; Watson et al. 2004). One such finding was the conclusion that cigarettes marketed as lower in nicotine yield ("lights" and "ultralights") had a greater percentage of free nicotine (Watson et al. 2004). Published reviews of internal documents also demonstrate that even "small" changes could significantly increase their ability to deliver an "optimum" dose of nicotine and result in distinct differences readily recognizable among smokers (Ferris Wayne et al. 2006; Hurt and Robertson 1998; Pankow 2001). Further, industry scientists exhibited a clear understanding that free nicotine alters the rate of absorption and physiological impact among smokers (Hurt and Robertson 1998).

2.4 Sensory Factors and Subjective Response

While reduction of harshness is a key component of tobacco product design, the smoker nonetheless relies on a combination of smoking cues, which affect smoking behavior as well as reinforce the physiological impact of nicotine (Rose 2006). Sensory cues, which include stimulus aspects of cigarette smoke, can elicit responses from the basic senses (sight, smell, touch, taste) as well as physiological responses from the nerve system, such as the olfactory and trigeminal nerves

(Carpenter et al. 2006; Rose 2006), and contribute to overall subjective response apart from nicotine delivery. Cigarette manufacturers dedicated tremendous resources to investigation of perceived sensory stimulation in order to understand and maximize the role of sensory effects in the smoking experience. For example, industry researchers recognized that sensory properties were linked with smoking behavior and puffing parameters produced by the smoker (British American Tobacco 1983, 1994, 2005; Morgan et al. 1990). An undated RJR document summarized:

> The consumer may alter his smoking behavior based on sensory information so as to modify the sensory, chemical, and physiological properties of the smoke. This has implications for the physiological and psychological effects of smoking, since they may be affected by smoke dose and composition (R.J. Reynolds 1999).

These findings are confirmed in the published literature. In a study comparing a high nicotine/high sensory cigarette, a low nicotine/low sensory cigarette, and a low nicotine/high sensory cigarette, subjects regulated their smoking behavior according to sensory intensity rather than nicotine intake (Levin et al. 1993). In another study, pharmacological effects from denicotinized and regular cigarettes were compared (Pickworth et al. 1999). While subjects preferred the regular cigarettes, both types of cigarettes reduced subjective measures of craving and withdrawal.

Free nicotine was believed by manufacturers to be a critical sensory component (Brooks et al. 1974; Hirji and Wood 1973; Hurt and Robertson 1998; Maynor and Rosene 1981; Pankow 2001). Free nicotine provides a more immediate impact or "kick" in the back of the mouth and throat, preceding the arrival of nicotine to the brain, with even a small amount of free nicotine discernible by the smoker (Hurt and Robertson 1998). As summarized in a 1976 memo: "As the pH increases, the nicotine changes its chemical form so that it is more rapidly absorbed by the body and more quickly gives a 'kick' to the smoker" (Mckenzie 1976). RJR funded studies in the late 1980s in an effort to understand trigeminal chemoreception in the nasal cavity, and concluded, "Nicotine is the most effective trigeminal stimulus, and perhaps the most irritating" (Silver 1988).

While nicotine contributes to the sensory aspects of smoking, other physical and chemical properties of tobacco smoke also provide rewarding sensory stimulation to smokers. As described by Rose (2006), the smoking-induced "sensory package" influences smoking behavior and dependence (Brauer et al. 2001; Carpenter et al. 2006; Rose 2006). A target of internal sensory research was the contribution of peripheral nerve responsivity to the total behavioral phenomenon (Philip Morris 1995; R.J. Reynolds 1987a). For example, a 1995 Philip Morris document described a series of research proposals to identify the sensory characteristics of nicotine and their relative contribution (alongside other stimuli) to smoker responses (Philip Morris 1995).

A related area of interest was the use of sensory stimulation to provide a bridge between product expectations and smoke delivery. As proposed by a Phillip Morris scientist: "We might be able to produce the CNS effects of high delivery cigarettes by leading subjects to believe they [are] smoking high nicotine cigarettes when they [are] actually smoking low nicotine cigarettes. Experiments of this type might have

important implications for the marketing of low delivery cigarettes" (Gullotta 1982). This research further highlights the importance of sensory effects on response to nicotine.

2.5 Behavioral Determinants of Nicotine Dosing

"Compensatory" smoking, described in much greater depth elsewhere in this volume, was recognized by the industry since the introduction of "light" cigarettes more than 30 years ago (National Cancer Institute 2001). A Philip Morris memorandum observed in 1968 that "since there is evidence that the smoker adapts his puff, it is reasonable to anticipate that he adapts to maintain a fairly constant daily dosage" (R.J. Reynolds 1997). Evidence to support this thesis was gathered in subsequent years by tobacco manufacturers (Dunn and Schori 1972b; Hurt and Robertson 1998; Phillip Morris 1974).

Manufacturers acted to take advantage of compensatory behavior that was driven by smoker's addiction to nicotine, and enhance it through cigarette designs that supported compensation. A 1977 BAT review observed:

> It is now possible to design cigarettes which would have the same smoking machine delivery but different deliveries to the compensating smoker. Broadly speaking, this could be achieved by developing cigarettes with a knowledge of the smoker's response to such factors as pressure drop, ventilation, irritation, impact, nicotine delivery, etc. (Haslam 1977)

A 1984 BAT document lists as "high priority" the development of "alternative designs (that do not invite obvious criticism) which will allow the smoker to obtain significant enhanced deliveries should he so wish" (British American Tobacco 1984b).

Internal documents also indicate that other product design factors could be used to alter smoking behavior. For example, smokers rate lower yield cigarettes as harder to draw because of the loss of sensory impact (Jeltema 1987), whereas the addition of an irritant makes it seem easier to puff (Walker et al. 1992). Internal studies investigated flavor discrimination of different compounds, with a particular goal of identifying olfactory responses with "feel" (mild irritant) qualities. This work led to the development of specific additives aimed at enhancing both the flavor and physical "feel" of tobacco smoke (Farnham 1995), as well as assisting development of better puff profile characteristics (Jennings et al. 1991).

The internal application of behavioral research to nicotine dosing is illustrated in a model taken from a 1984 BW document (see Fig. 3; from Ayres and Greig 1984). BW researchers developed a methodology designed to measure the "smoking dynamics" of a cigarette utilizing a reward-for-effort model, in which effort was the work done to obtain a volume of smoke and reward was the perceived delivery. This model was used to relate specific product changes to subjective product attributes affecting smoker response (Ayres and Greig, 1984). Thus, behavioral determinants of smoke nicotine yield were incorporated alongside product characteristics to model expected delivery and to aid in development of new products.

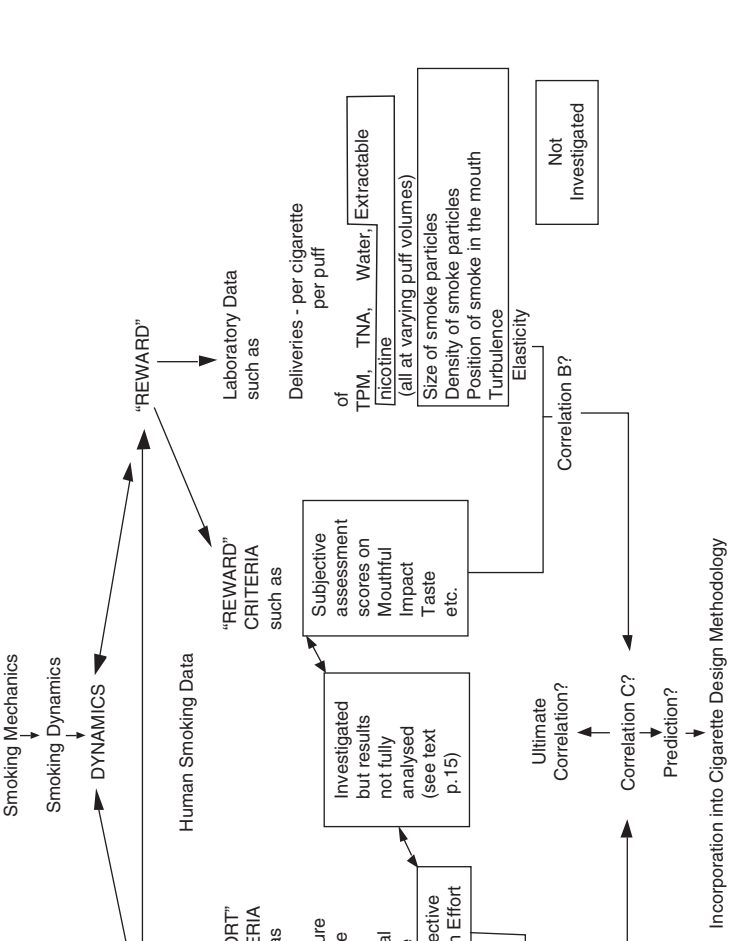

Fig. 3 Internal BW model: "An outline of those parameters thought to be involved in 'effort' and 'reward', and showing those experimentally accessible and examined" (Ayres and Greig 1984)

2.6 Consumer Targeting and Differences in Smoker Response to Nicotine

Unique product characteristics (e.g., nicotine yield, draw, length, sensory impact) may reflect real differences in the wants and needs of individual smokers and groups. A goal of internal psychology research was to use identified differences in personality types, motivations, and needs to enhance product segmentation and brand differentiation. For example, a number of manufacturers sought to relate personality and psychological profiles to specific brand attributes, such as nicotine, tar, taste, and cigarette draw (Cook et al. 2003).

For example, BAT tested the hypothesis that groups of smokers with a high "inner need" score would reject low nicotine cigarettes, whereas groups with a low inner need score would find low nicotine cigarettes acceptable and prefer them to cigarettes with higher nicotine delivery. "Inner need" was defined by BAT scientists (Wood 1974) as the extent to which smokers use cigarettes to fulfill individual needs (e.g., concentration; relieve nervousness, anxiety or boredom; relaxation; substitution for eating sweets) and contrasted with a "social" dimension (i.e., extent to which cigarettes are used to conform with social groups, etc) (Wood 1974). The hypothesis was confirmed in that low need smokers preferred to smoke cigarettes with 1.0–1.5 mg of nicotine, while high need smokers preferred to smoke 1.5–2.0 mg nicotine cigarettes (Wood 1976). Similarly, the nicotine RSM (response surface methodology) study, conducted throughout the 1980s and early 1990s by RJR, clustered smokers into categories based on their motivations to smoke and their personality characteristics (R.J. Reynolds 1979). These and similar internal studies are described in Cook et al. (2003).

Internal research also confirmed differences in the effects of nicotine by gender and age. BAT contrasted "sensory pleasure" (i.e., taste and enjoyment) as the major component of female preference, with "satisfaction" (i.e., presumably nicotine delivery) as the key factor among males (Barton 2000). Other internal studies found differences in compensatory behavior among male and female smokers, suggesting that since female smokers inhale less and experience reduced nicotine intake, they may be less influenced by the pharmacological affects of nicotine but may "use ritual of lighting and puffing on cigarettes to calm themselves under stressful situations" (Comer 1977). These findings were then reflected in product differences. Tar and nicotine levels were manipulated to achieve product benefits consistent with female smoking patterns (Fleming 1986; Potter 1991). BAT proposed development of a new female brand that would deliver sensory pleasure in direct response to this issue (Barton 2000). The general emphasis on low delivery products targeting women reflects internal research regarding the reduced importance of nicotine among female smokers.

Cigarette manufacturers likewise demonstrated in internal research that sensory perceptions are unique for younger and beginner smokers due to low tolerance for irritation and an "undeveloped" taste for tobacco smoke. Products were tailored to presmokers or learners with bland, soft, moist mouth-feel, and minimal irritancy,

harshness, or astringency (Teague 1973). RJR analyzed the characteristics of cigarettes that had become popular historically among younger smokers, and concluded that the most important physical characteristic of the younger adult brand was its smoothness or mildness (Wayne and Connolly 2002).

3 Methods for Manipulating Nicotine Dosing

The conventional cigarette is relatively uniform, and many basic product differences are clearly defined by well-known market categories (e.g., length, circumference, "menthol"). However, Connolly and others have demonstrated that additional product differences introduced by manufacturers may alter smoke chemistry, mechanisms of delivery, perception of smoke, bioavailability, and smoker behaviors (Carpenter et al. 2006; Cook et al. 2003; Ferris Wayne and Connolly 2004; Keithly et al. 2005; Wayne and Connolly 2002). For example, the basicity of smoke ("smoke pH") may be controlled by tobacco processing and other changes, altering the route or speed of chemical uptake (Henningfield et al. 2004b; Hurt and Robertson 1998). Likewise, new smoke compounds may be introduced or enhanced, including nicotine analogs and synergists, and bronchodilators (Bates et al. 1999; Wayne et al. 2004).

Tobacco manufacturers have successfully developed and utilized physical and chemical product design changes to control the quantity, form, and perception of nicotine dose. Methods included altering physical construction parameters, such as the tobacco, filter, ventilation, and paper; altering smoke chemistry, for example by increasing smoke pH through addition of ammonia or other bases; and introducing new smoke compounds to increase the potency of nicotine. Consequently, these factors must be taken into account in assessment of nicotine dosing. In addition, other features of the product (e.g., brand name, packaging, image, and advertising), not described in this chapter, also play an important role in influencing consumer acceptance and smoking effects and warrant research attention.

3.1 Physical Cigarette Construction Parameters

Different tobacco types (e.g., flue-cured, Virginia tobacco; air-cured, Burley tobacco; sun-cured, Oriental tobacco) and leaves from different stalk positions affect the composition of cigarette smoke and the delivery of nicotine (as well as sugars and other constituents) to the smoker. For example, cigarettes that contain air-cured Burley tobacco (a major constituent of American blend cigarettes) produce greater smoke nicotine yields and a higher proportion of "free" nicotine, whereas oriental Turkish-type cigarettes deliver substantially less nicotine, nearly all in the protonated or bound form (Bernasek et al. 1992). Consumption of some European

cigarettes containing only Burley tobaccos can maintain blood nicotine levels with minimal smoke inhalation, similar to cigars.

In the 1980s, BAT and BW developed a tobacco referred to as "Y-1," which was genetically engineered to have a nicotine content approximately twice that of conventional tobacco. This nicotine-enhanced tobacco was blended with other tobaccos in order to alter nicotine-to-tar ratios in commercial cigarettes sold in the USA (Chakraborty 1985). Thus, differences in blend can produce significant variations in nicotine concentration in the tobacco rod, supporting differences in smoke composition and smoke yield. In Connolly et al. (2007), one of the design features that best defined smoke nicotine yield was the concentration of nicotine in the tobacco rod. Significant increases over time were observed both in the concentration (9%) and total nicotine content (17%) in the tobacco rod, suggesting differences in blend.

Other physical characteristics such as length, circumference, porosity, ventilation, and tobacco weight and density combine to determine the basic machine-smoked yields of "tar", nicotine, and other substances. The complex interaction between these different design features has been extensively studied within the tobacco industry in order to carefully control the resulting product delivery (Browne 1990).

Product design characteristics may also affect the ability of the smoker to self-regulate dosage, and are therefore introduced by the manufacturer and used by the smoker to control delivered smoke yields (Norman 1974, 1983). For example, an RJR review of 17 internal studies, relating cigarette construction parameters to observed sensory properties, concluded that a number of cigarette design features play a significant role in determining how a cigarette is smoked – with key factors being air dilution, draw resistance, and filtration efficiency (Roberts 1985). A Lorillard presentation on development of "low-yield" cigarettes describes the use of draw resistance as follows:

> . . . the puff volume that the smoker extracts from a cigarette is a function of the resistance that he encounters in the cigarette unlike in the FTC smoking regime where the puff volume is constant regardless of draw resistance. . . . If the object is to design a cigarette that has a very low FTC tar but that tastes like a 6 or 7 mg cigarette then the pressure drop distribution in the cigarette has to be manipulated in such a fashion that the smoker can draw larger than the standard FTC 35 cc 2 second puffs and still remain within his comfortable smoking effort range (Norman 1983).

Specific product changes proposed include the use of ventilation, filtration, and tobacco rod density to alter draw resistance (Norman 1983; Thorne 1994); the introduction of channeled or other unique filter designs to enhance sensory properties such as sensations in the mouth, referred to as "mouthful feeling" (Brown & Williamson 1983; Greig 1987; McMurtrie and Silberstein 1980); and the use of higher nicotine tobaccos, flavor additives, and alkaline additives to increase a range of sensory attributes (Shepperd 1993; Whitehead 1994).

Of all the design characteristics of cigarettes, filter ventilation may be the most important in determining machine-smoked yields (Djordjevic et al. 1995), as well as the most important determinant of the differences in machine-smoked yields and human smoking behavior and smoke exposure. Filter ventilation dilutes mainstream

smoke with air. Consequently, the rod characteristics become less important determinants of yield in the presence of filter ventilation (Schneider 1992).

A recent study demonstrated that increases in filter ventilation increased the relative proportion of free-base nicotine in the mainstream smoke when measured in an unblocked machine-smoked condition (Watson et al. 2004). The practical importance of these results is that, even without any compensatory smoking behavior, a ventilated cigarette may deliver a greater proportion of total nicotine in free base on a puff-by-puff basis.

3.2 Smoke Composition and Product Chemistry

Reconstituted tobacco is used at high levels in most American blend cigarettes, which are popular in many regions of the world (National Cancer Institute 1996). Reconstituted tobacco sheets are made from processing stems and other parts of the tobacco leaf that would otherwise go to waste. In the course of manufacturing reconstituted tobacco, numerous chemicals are added, including nicotine as replacement for the amount lost in the manufacturing process (Browne 1990; National Cancer Institute 1996). Indeed, this also provides an effective means for manufacturers to control or even increase the amount of nicotine in the total blend (Minnemeyer 1977).

Ammonia compounds are a primary chemical component of many reconstituted tobaccos. The importance of ammoniation in the development of the characteristic flavor popularized by Marlboro has been widely publicized (Bates et al. 1999; Freedman 1995; Hurt and Robertson 1998). The chemical impact of ammoniation is complex and appears to influence the form and delivery of nicotine in a variety of interconnected ways (see BW Fig. 4) (Johnson 1989). Ammoniated reconstituted tobacco has a characteristic mild sensory profile, and features a number of important compounds created through the reaction between ammonia and sugars (J.R. Reynolds 1980; Wells and Kendrick 1995). Addition of ammonia as a strong base leads to increased smoke pH, which corresponds with increased levels of free nicotine in smoke (Hurt and Robertson 1998). Thus, a 1982 position paper from RJR observed that "... ammonia in smoke is one of the major pH controlling components" and that "... studies of the effect of ammonia on smoke composition showed... an increase in physiological satisfaction with increasing ammonia content" (Bernasek and Nystrom 1982).

Ammoniation also improves sheet tobacco strength and facilitates nicotine scavenging from the remaining cigarette tobacco, thus increasing the transfer efficiency of nicotine to smoke (British American Tobacco 1988; Wells and Kendrick 1995). Indeed, a recent review of the literature concluded that changes in nicotine kinetics (shorter $t_{1/2}$; higher C_{max}) due to ammoniation leads to higher concentrations of nicotine in mainstream smoke rather than faster absorption of nicotine in the pulmonary tract (Willems et al. 2006).

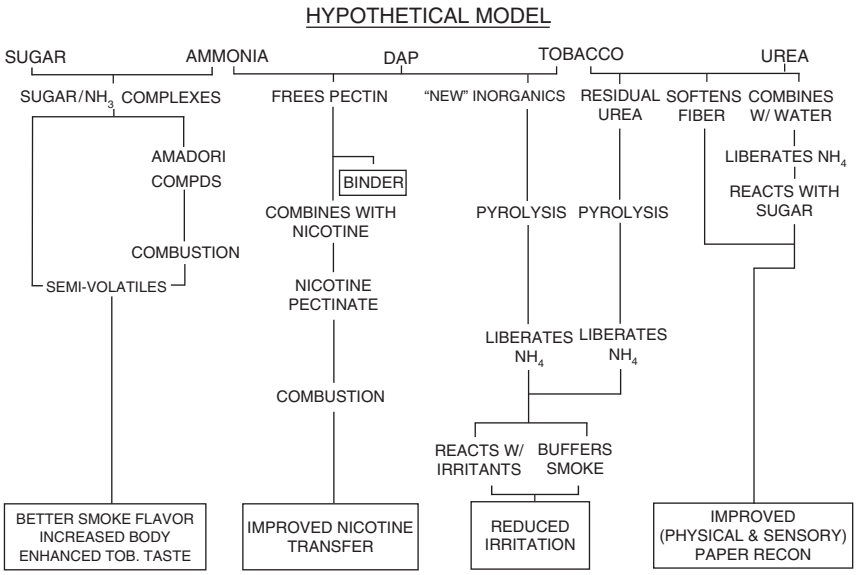

Fig. 4 Industry model of the effects of ammonia on form and delivery of nicotine (Johnson 1989)

Although ammoniation is the most commonly cited example of industry manipulation of smoke chemistry, the internal documents describe countless others. For example, sugars play a critical role in combination with ammonia in the formation of pyrazines and other compounds via reaction processes (Harllee and Leffingwell 1978; Swain and Crayton 1981; Wu and Swain 1983). Balancing the levels of sugars and nicotine may lower smoke pH and reduce levels of free (unprotonated) nicotine, which also correspond with reduced harshness and impact (Bernasek et al. 1992; R.J. Reynolds 1992b; Smith 1992). Evaluation of smoke chemistry often focused on a combination of variables; sugars, for example, were combined with amino acid mixtures to alter smoke flavor (Crellin and Reihl, 1984). A 1992 RJR document states: "Based on initial testing it is highly likely that efforts in the area of sugar/nicotine balance will provide incremental improvements in the area of smoothness and harshness" (R.J. Reynolds 1992a). Industry scientists considered sugar/nicotine technology particularly important in the development of full-flavor low tar products.

Other methods explored internally to alter the form of nicotine delivered included the use of base- or acid-coated filters. For example, researchers at RJR applied sodium hydroxide-coated filters to a cigarette yielding only 0.06 mg of nicotine in order to heighten sensory impact (Shannon et al. 1992). Alternately, a filter coated with an acid (lactic, levulinic, citric) was used to reduce the impact of a high nicotine sheet, either by trapping the nicotine or by changing the pH of the smoke so there is not as much nicotine in the vapor phase (Shannon et al. 1992). The researchers noted that:

> Lactic and levulinic acids get into the smoke and they have a bigger smoothing effect than citric acid, which we are pretty sure does not get into the smoke. It does not smooth so much. In one case you have got both things going for you. You are restricting nicotine vapor plus you are dumping acids into the smoke. In the other case you are just absorbing nicotine vapor and you don't get as much of the nicotine (Shannon et al. 1992).

Similarly, in a study titled "When nicotine is not nicotine," Philip Morris researchers observed that cigarettes that had been oversprayed with nicotine citrate were half as effective in nicotine transport and delivery when compared with those sprayed with nicotine as the base; the latter were perceived as having higher mouth/throat impact (Gullotta et al. 1989).

The ratio of nicotine to other compounds delivered in smoke (e.g., nicotine/tar ratio) may also be a significant determinant of response. Philip Morris conducted multiple consumer research studies through the late 1970s to determine the acceptability of various nicotine/tar ratios, and found that consumers preferred nicotine/tar ratios that were higher than those that occur naturally in tobaccos (Dunn and Schori 1971, 1972a; Dunn et al. 1973, 1976; Houck et al. 1975, 1976). RJR pursued a project during this same time period "to determine means to manipulate nicotine/tar ratio to provide a more satisfying smoke" (Henley 1977). Methods to increase the nicotine/tar ratios included blend modifications (i.e., the use of high nicotine tobaccos), the direct addition of nicotine to the blend, and addition of a "nicotine salt complex… to increase the nicotine delivery" (R.J. Reynolds 1985, 1990a). A 1990 RJR document describing "Project XB" states that: "Nicotine is a key element in providing acceptable taste and satisfaction. Nicotine itself is harsh and irritates. High nicotine tobacco salts allow increase in smoke nicotine yields without corresponding increases in harshness attributes" (R.J. Reynolds 1990a).

3.3 New Smoke Compounds

Cigarette manufacturers may reinforce the physiological effects of nicotine, or otherwise influence the response to nicotine, through introduction and use of compounds that interact with nicotine but do not directly alter its form or delivery.

Manufacturers have acknowledged the use of hundreds of chemical additives in tobacco products (Leffingwell et al. 1972; Philip Morris 1994). Some of these additives may synergize with nicotine or demonstrate other reinforcing effects. Cocoa, for example, contains alkaloids, which may themselves have pharmacological effects when inhaled, or may modify the effects of nicotine. Pyridine is chemically a portion of the nicotine molecule, acting as a CNS depressant similar to nicotine, although it is less potent.

When burned, sugars also increase the smoke levels of acetaldehyde, another potential nicotine reinforcing agent. Recent studies demonstrate that acetaldehyde enhances behavioral and neuronal responses to nicotine in both adolescent and adult rats (Belluzzi et al. 2005; Cao et al. 2007). In the early 1980s, DeNoble and coworkers at Philip Morris studied the behavioral effects of nicotine and acetaldehyde in

rats. Results of this research showed that the two compounds work synergistically, producing greater addictive effects. DeNoble claims that once this information was obtained, Philip Morris increased the level of acetaldehyde in Marlboro cigarettes by 40% between 1982 and 1992 through the addition of sugar (Bates et al. 1999).

Another area of research was the identification of analogs and synergists that could be used to imitate or alter the response to nicotine. Industry research identified a number of analogs internally that could be used to imitate or enhance the response to nicotine (Vagg and Chapman 2005; Wayne et al. 2004). RJR's analog research tested multiple potential nicotine analogs to compare with nicotine's effect on cholinergic receptor systems in the brain, cardiovascular activity and pharmacological potency, and cardiovascular and behavioral endpoints. In 1989, a RJR progress report indicated that 29 compounds competed for nicotine binding to receptors at physiologically relevant concentrations, based on relative potencies (Wayne et al. 2004).

Ferris Wayne and Connolly, (2002) assessed the potential effects of menthol as an additive in cigarettes. Their findings linked menthol to a range of unique physiological and subjective effects in cigarettes, including reduced harshness, increased nicotine-associated impact, greater ease of inhalation, and enhanced bioavailability of nicotine. Menthol has been shown to exhibit absorption-enhancing effects, as well as having possible effects on drug metabolism that could alter the pharmacological action of other substances in tobacco smoke (Jori et al. 1969; Madyastha and Srivatsan 1988; Ferris Wayne and Connolly 2004).

Levulinic acid provides another example of the multiple influences that additives can demonstrate with respect to nicotine dose. RJR scientists generated considerable evidence that levulinic acid and nicotine levulinate decreased harshness and increased nicotine yield from tobacco smoke. Further, internal research demonstrated the capacity of levulinic acid to enhance the binding of nicotine in the brain, increasing its pharmacological effectiveness. The addition of levulinic acid also altered the composition of mainstream and sidestream smoke and introduced potentially toxic pyrolysis products to smoke. These combined effects could have significant implications for the increased addictiveness or toxicity of the cigarette and for the progression of smoker behaviors, including initiation and quitting. Measured increases in peak plasma nicotine levels among treated low-yield cigarettes confirm the importance of these internal findings in the assessment of brand deliveries. (see Table 1, from Keithly et al. 2005)

4 Implications for the Commercial Market

Both independent studies and internal industry research highlight the relevance of product differences in determining nicotine dosing. Historical differences are indicated, as are differences among brands. The evidence demonstrates the effectiveness of methods adopted by manufacturers to control nicotine dosing and target the needs of specific populations of smokers through commercial product development.

Table 1 Summary of effects of levulinic acid when used as an additive in cigarettes

Potential effect	Measure used	Objective	Outcome
Sensory perception (Steele 1989)	Subjective consumer panel testing of treated cigarettes	Offset harshness and irritation in development of low-yield cigarettes	"…physiological clues are being blocked, since people smoked the test cigarettes essentially the same as the control, even though more nicotine was obtained"
Smoke pH (Stewart 1988)	Electrode-based measure of smoke pH of machine-smoked cigarettes	Offset harshness and irritation in development of low-yield cigarettes	Decreased smoke "pH" in Camel Light when used alone or added as nicotine levulinate
Nicotine delivery in smoke (Steele 1989)	Puff profiles of smokers, followed by yield measures based on Human Mimic Smoking Machine	Increase smoke nicotine delivery of low-tar cigarettes relative to higher tar controls	Smoke nicotine delivery increased in a number of tested brands, including Camel Light, Vantage Ultra Light, and Now
Plasma nicotine (Steele 1989)	Plasma nicotine change from baseline measured comparing smokers of control and test cigarettes	Increase bioavailability of smoke nicotine in low-yield and low tar/nicotine cigarettes	Plasma nicotine for Now about 6 ng ml^{-1} higher than the control (Winston King); for Winston Ultra Light, about 13 ng ml^{-1} higher than control
Tar/nicotine ratio (Steele 1989)	Machine-measured smoke yields of tar and nicotine	Increase delivery of nicotine relative to tar in low-yield cigarettes	Levulinic acid alone had little effect; nicotine levulinate decreased tar and nicotine by half in Winston Ultra Light and Now
Receptors in brain (Steele 1989)	In vitro studies on binding of radiolabeled nicotine to pharmacological receptors in brain tissue	Determine whether levulinic acid affects nicotinic cholinergic receptors in the brain	Observed increased nicotine binding ranging from 20–50%, with a mean value of around 30%; "…changes in receptor binding may lead to changes in physiological effects of nicotine…"

4.1 Historical Trends

Over the last several decades, manufacturers have significantly decreased tar and nicotine yields. Between 1954 and 1993, the average sales-weighted "tar" and nicotine yields of US cigarettes have declined from 38 and 2.7 mg to 12 and 0.9 mg, respectively. (Hoffman et al. 1997) These changes have resulted from product design changes as discussed previously. Tar/nicotine ratios have also declined, resulting in

more nicotine per unit of tar. In 1979, BW researchers found that the average nicotine/tar ratio for commercial brands had increased, and that the nicotine/tar ratio for low yield products was higher than for regular products (Esterle et al. 1979). Internal studies indicate that this decline was intentional and competition driven. RJR stated in 1990: "PM has successfully raised the nicotine levels on all their products (across the line) by using high nicotine tobaccos. Thus, they already have a better T/N ratio than their competitors. Adding nicotine could further improve that ratio" (R.J. Reynolds 1990c). RJR continued its own research into changing the nicotine/tar ratio throughout the 1990s, concentrating on development of an ultralow tar product that provided nicotine delivery similar to full-flavor products (Wilson 1991).

A published study (Wayne and Connolly 2002) on product changes in the Camel brand found an increased trend in nicotine levels between 1989 and 1994, with simultaneous reduction in tar/nicotine ratios. These changes corresponded with Camel's increase in popularity among youth smokers. Although increased nicotine delivery is typically associated with increased throat impact and irritation, product attribute measures for Camel brand styles between 1988 and 1991 demonstrate how increases in nicotine were offset by product changes targeting smoothness and harshness. The findings suggest that changes in tar/nicotine ratios may have offset the harshness commonly accompanying an increase in nicotine delivery, supporting increased intake of smoke nicotine, particularly among new smokers unaccustomed to nicotine.

4.2 Brand Differences

Internal findings provide valuable comparisons between cigarettes, both with respect to design and delivery as well as for responses among smokers. For example, a 1986 study at RJR assessed "quenching" desire for a cigarette among human smokers. Camel Light and Marlboro Light were comparable in quenching, and both were less than Winston and Marlboro, which were also comparable; Now cigarettes "quenched" desire less than all other brands (R.J. Reynolds 1986).

RJR's Winston/Marlboro study assessed inhalation and uptake among smokers, and demonstrated that the smoke nicotine of Winston smokers decreased as puff volume increased, while the opposite relationship existed for Marlboro smokers – a phenomenon thought to be explainable by differences in the amount of "wasted" smoke (Fluhler 1992). A Philip Morris study of low-yield cigarettes confirmed differences in subjective response, and noted: "The electrophysiological results suggest that extended testing might expose subjective differences among low delivery cigarettes that are not readily apparent in single cigarette comparisons" (Gullotta et al. 1990b).

RJR researchers discussed differences among their products and those of competitor Philip Morris. They observed that the Marlboro has less smoke nicotine than Winston, but a higher level of weaker bases, such as pyrazines. These weaker bases

"accounted for the pH being slightly higher" of Marlboro – and equivalent levels of volatile or "free" nicotine – despite the fact that nicotine was not as high. One difference that the researchers noted with respect to Philip Morris products was that following the initial production of reaction products (via ammoniation), their products remained extremely consistent over time. By contrast, the process used by RJR "did not quench that reaction and that product continued to change over time" (Shannon et al. 1992).

Ferris Wayne et al. (2006) observed consistent relationships among brands, for internal smoke pH and free nicotine measurements, supporting the utility of these measures in brand comparisons. For example, during the period 1965–1980, Marlboro exhibited consistently higher smoke pH values relative to Winston, regardless of testing method or manufacturer; after 1980, both brands gave similar smoke pH values. Similarly, "low yield" brands such as Merit and Barclay exhibited higher values relative to "regular" brands. Consistent patterns of relative brand differences such as these explain the long-term use of smoke pH measurements by the tobacco industry, and contrasts with public claims by the industry that little or no variation in smoke pH exists among brands (Philip Morris 1997, 1998). Internal results obtained for low yield brands are also consistent with the view that such brands may compensate for reduced total nicotine delivery by maintaining a certain level of free-base nicotine delivery.

5 Conclusions

Historical evidence with respect to manipulation of nicotine is overwhelming. Tobacco manufacturers began programs for manipulation of nicotine more than 50 years ago and refined these efforts over decades, altering product characteristics in order to sustain addictive levels of nicotine delivery, despite reduced machine-measured levels of tar and nicotine delivery.

In the August 2006 decision of a suit between the US federal government and major US cigarette manufacturers, federal Judge Gladys Kessler concluded that tobacco manufacturers:

- "... designed their cigarettes to precisely control nicotine delivery levels and provide doses of nicotine sufficient to create and sustain addiction."
- "... extensively studied smoking intake and inhalation, compensation, addiction physiology, smoker psychology, the pharmacological aspects of nicotine, the effects of nicotine on brain waves, and related subjects."
- "... intentionally developed and marketed cigarettes which, in actuality, delivered higher levels of nicotine than those measured by the FTC method."

There is no evidence to suggest that this historical pattern of nicotine manipulation has changed. Numerous product adjustments are used to optimize both levels of nicotine as well as effects. Manufacturers have concealed much of their nicotine-related research, and have continuously and vigorously denied their efforts

to control nicotine levels and delivery. Nonetheless, the modern cigarette reflects many decades of sophisticated internal research on nicotine dosing, incorporating sensory, behavioral, psychological, and social factors alongside a highly engineered chemical delivery system designed to increase ease of use, enhance the physiological effects of smoking, and most effectively match the needs of smokers.

Although the major focus of this review has been on the manipulation of nicotine dosing characteristics of cigarettes, it is also clear that product design and ingredients facilitate tobacco addiction through diverse addiction-potentiating mechanisms. This conclusion is similar to that of Buchhalter and colleagues (see chapter in this volume) and others (Henningfield et al. 2004a, b; WHO 2007) that, in addition to designs and ingredients that enhance nicotine self-administration and absorption (e.g., filter tip ventilation, menthol, and levulinic acid), ingredients may have their own direct pharmacologic effects that potentiate those of nicotine (e.g., acetaldehyde) and may increase the free-base fraction of nicotine (e.g., ammonia and urea-based compounds). Still other designs may increase the attractiveness of the product through the illusion of reduced harmfulness and even candy-like flavorings. These observations are also consistent with the conclusion that tobacco products in general, and cigarettes in particular, though addictive by nature, carry enhanced addiction risk through modern designs that were intended to achieve this affect (US Federal Drug Agency 1995, 1996; Henningfield et al. 2004a, b; WHO 2007).

Results of the few laboratory studies conducted with bidis and clove cigarettes (highly flavored and unique tobacco products) indicate that in spite of large differences in the availability of nicotine in these products, experienced smokers will extract quantities of nicotine similar to those extracted when smoking their usual brand of cigarettes. However, to the extent that cigarette designs make addictive levels of nicotine delivery easier to achieve, such designs may facilitate the path to addiction for youth or new smokers, and enable smokers to sustain their addiction in the face of smoking restrictions, increased prices, or other obstacles.

Evidence supports the need for regulatory oversight of both nicotine and tobacco (as its delivery mechanism). Cigarette manufacturers have dedicated extensive resources to careful investigation of nicotine manipulation in order to understand and optimize the role of nicotine in the smoking experience. Regulation of tobacco products is needed to assess product changes that are used to reinforce or contribute to tobacco dependence. Assessment should include not only levels of nicotine in smoke, but also factors known to influence dose and effects. Among these are the form of delivery of nicotine, potential nicotine analogs or synergists, the role of “impact” or other cues, and physical or chemical design differences (e.g., ventilation) likely to influence puffing and inhalation behavior.

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Pharmacotherapy for Tobacco Dependence

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Contents

Abstract Pharmacotherapy can provide effective treatment of tobacco dependence and withdrawal, and thereby facilitate efforts to achieve and sustain tobacco abstinence. Currently approved medications for smoking cessation are nicotine replacement medications (NRT), including nicotine patch, gum, lozenge, sublingual tablet, inhaler and nasal spray, the antidepressant bupropion, and the nicotinic partial agonist varenicline. This review discusses the pharmacological basis for the use of these medications, and the properties that might contribute to their efficacy, safety, and abuse liability. The review also discusses how pharmacological principles can be used to improve existing medications, as well as assist in the development of new medications.

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1 Introduction

Although many people are able to quit smoking without formal intervention, this generally occurs only after many cessation attempts, and after sufficient harm has been done to substantially increase the risks of premature mortality (Royal College of Physicians 2000; US Department of Health and Human Services 2000). For others, achieving remission from dependence without treatment is much more difficult, if not practically impossible, largely because of the pathophysiological changes in brain structure and function produced by long-term exposure to nicotine and possibly to other substances in tobacco that contribute to the persistence of tobacco use.

Pharmacological aids to smoking cessation can be life-saving, because they generally double the probability of successful tobacco abstinence, enabling more people who may have been previously unsuccessful at quitting to achieve lasting abstinence. Pharmacotherapy is also among the most cost-effective forms of medicinal therapy (World Bank 1999; Parrott et al. 1998). The extant variety of medications is important because of differences across individuals in acceptability (e.g., patch versus gum versus lozenge form of nicotine replacement) and differences in mechanism of action that might confer advantages for at least some populations (e.g., antidepressant versus nicotinic partial agonist). In addition, clinical practice guidelines (e.g., Fiore et al. 2000) endorse evidence-based variations from medication use according to approved labeling, to address a more diverse range of patient needs. These observations suggest a need to expand the range of persons who can be effectively treated by pharmacotherapy. Hopefully, this can be accomplished by continued development of more effective strategies for use of currently available medications, and the development of new medications that will be effective for persons who appear refractory to current treatments. This article is intended to support treatment development by reviewing effective treatments and their apparent mechanisms of action, as well as implications for development and application of medications.

1.1 Treatment Goals and Mechanisms of Action

What is commonly referred to as tobacco addiction or tobacco dependence has been clinically delineated into two specific diagnosable disorders: dependence and

withdrawal (American Psychiatric Association 2000; World Health Organization 1992). Dependence refers to the maladaptive, chronic, and typically relapsing use of tobacco that meets the same types of criteria that are applied to other forms of drug dependence. Withdrawal refers to the generally time-limited syndrome that occurs upon termination of drug use, is varied across drug classes, and is frequently, but not always, present in dependent persons. For tobacco, withdrawal symptoms include anxiety, anger, difficulty in concentrating, sleep disturbance, and weight gain (Hughes and Hatsukami 1986). Powerful recurring cravings are also prominent.

Presently, the primary recognized outcome upon which medications are approved by the US Food and Drug Administration (FDA) and most other medical regulatory bodies worldwide is to aid smoking cessation, and this outcome is the primary focus of the present article. The mechanisms of medication action that support smoking cessation appear to vary across treatment approaches, and may include reduced withdrawal symptoms including cravings and cognitive deficits, reduced reinforcing effects of smoking, and provision of at least some of the benefits derived by smoking, such as control of mood and appetite control. For example, as discussed in the next section, nicotine replacement medications reduce withdrawal symptoms by partially replacing the nicotine normally provided by smoking. Antidepressants such as bupropion and nortriptyline may be efficacious for smoking cessation, in part because of their reduction of the cessation-induced depression that is related to nicotine withdrawal.

The high prevalence of psychiatric comorbidity associated with cigarette smoking is notable. Nicotine-dependent individuals with a comorbid psychiatric disorder make up 7% of the population, yet consume 34% of all cigarettes smoked in the USA (Grant et al. 2004). Specific examples of cooccurrence are numerous. One study found that rates of current daily smoking among psychiatric inpatients were 83% for patients with schizophrenia and 65% for patients with mood disorders, compared to 26% for community controls (de Leon et al. 2002). Among adolescents, those with clinically significant ADHD symptoms were 2.8 times more likely to be daily smokers than those who did not display these symptoms (Tercyak et al. 2002). These types of findings may have implications for understanding the underlying behavioral and neurological mechanisms of nicotine dependence. Certainly, they highlight subpopulations that are in particular need of treatment for tobacco dependence.

There are a number of neural mechanisms by which a medication may alleviate withdrawal symptoms, simulate some of the reinforcing effects of nicotine, or block the reinforcing effects of nicotine. The most obvious is the nicotinic receptor itself, where nicotine replacement medications act. However, given that many of the effects of nicotine in the brain are likely mediated through modulation of a range of neurotransmitters such as acetylcholine, dopamine, glutamate, GABA, norepinephrine, and serotonin (Picciotto 1998), one might be able to mimic or block some of the reinforcing effects of nicotine by selectively activating or blocking these neurotransmitters. Moreover, the effects of acute and chronic nicotine on upregulation and desensitization of the nicotinic acetylcholine receptor (nAChR) are subjects of active research, the outcomes of which may have implication for the mediation of

medication effects (Buisson and Bertrand 2001; Buisson and Bertrand 2002; Gentry et al. 2003; Marks et al. 1992, 1993; Quick and Lester 2002; Rowell and Wonnacott 1990).

As discussed by Lerman et al. (2007), there are a variety of animal and human models that have been proposed to facilitate the drug development process, which include models that study the positive reinforcing and rewarding effects related to nicotine dependence, as well as the negative-reinforcing effects (i.e., alleviation of tobacco withdrawal). Models of positive reinforcing and rewarding effects include models of self-administration, drug reward, and conditioned reinforcement/reward. Negative reinforcement models include models of withdrawal and relapse (rodent models of nicotine physical dependence and withdrawal are reviewed in detail elsewhere; see the chapter by Malin and Goyarzu, this volume). In addition, drug discrimination models can be used to further examine the effects of potential medications for smoking cessation (e.g., whether a drug produces effects similar to nicotine), or reduce the discriminative stimulus properties of nicotine.

The following sections review the available pharmacologic therapies for smoking cessation, and discuss potential targets for the development of new medications.

2 Nicotine Replacement

The most direct way to help people manage the symptoms of nicotine dependence and withdrawal is therapeutic use of nicotine replacement therapy (NRT) (Fiore et al. 2000; Henningfield 1995; American Psychiatric Association 1996). Nicotine has been shown to be the main active ingredient in tobacco that causes and sustains addiction to tobacco (US Department of Health and Human Services 1988). Laboratory research has demonstrated that animals (Goldberg et al. 1983) and humans (Henningfield et al. 1983) who have been chronically exposed to nicotine or tobacco smoke will self-administer nicotine infusions. It should be noted that other constituents in tobacco, such as MAO inhibitors (Fowler et al. 1996a, b), may also play a role in tobacco dependence. The potential role of alkaloids other than nicotine has not been ruled out. This is consistent with the observations that what has been termed ‘tobacco delivered nicotine’ is more addictive and toxic than formulations provided by nicotine replacement medications (Royal College of Physicians 2000).

Nicotine administration has been shown to reverse the nicotine withdrawal seen upon discontinuation of chronic nicotine exposure in rats (Malin et al. 1992) and humans (West et al. 1984; Henningfield et al. 1986). Nicotine medications make it easier to abstain from tobacco by replacing, at least partially, the nicotine formerly obtained from tobacco. This provides nicotine-mediated neuropharmacologic effects, such as activation of dopaminergic reinforcement systems in the brain (US Department of Health and Human Services 1988). At the time of the early development of NRTs, substitution therapy using methadone had been used for decades as a treatment for opioid dependence (Charnaud and Griffiths 1952; Kreek 1979), with the understanding that partial blockade of opioid reinforcement was one aspect of

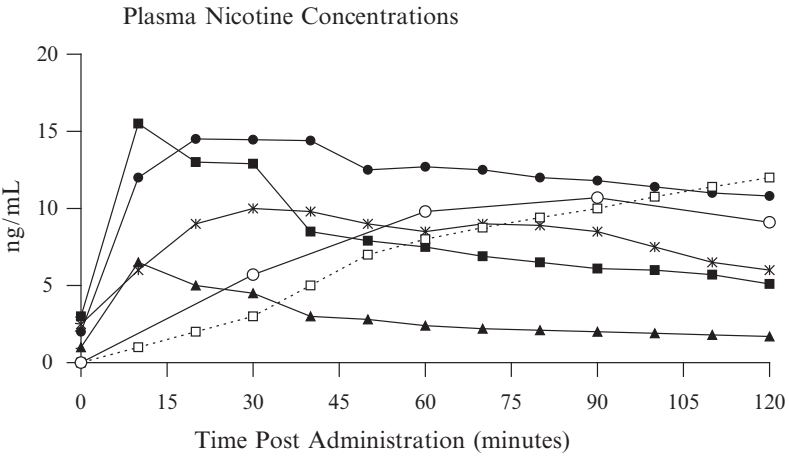

Fig. 1 Venous blood concentrations in nanograms of nicotine per millimeter of blood as a function of time for various nicotine delivery systems. Data on the cigarette delivering about 1 mg nicotine (*filled square*), oral snuff delivering 3.6 mg (*filled circle*), and 4 mg nicotine gum (*star*) are from those published by Benowitz et al. (1988). Data on 1 mg nicotine nasal spray (*filled triangle*) are from Schneider et al. (1996). Data on 21 mg transdermal nicotine patch (*open square*) are from Benowitz (1993). Data on the 4 mg nicotine lozenge (*open circle*) are from Choi et al. (2003)

methadone's mechanism of action (Donny et al. 2002). However, nicotine infusion has only modest effects on self-administration at concentrations typically provided by replacement medications (Benowitz et al. 1998; LeSage et al. 2003). Thus, it does not appear that blockade of nicotine reinforcing effects is a primary mechanism of action for NRT at doses commonly employed.

Currently approved NRT products include the transdermal nicotine patch and several acute NRT products, including nicotine gum, lozenge, sublingual tablet, vapor inhaler, and nasal spray. The single-dose nicotine plasma curves for transdermal patch, gum, nasal spray, lozenge, as well oral snuff and a cigarette, are illustrated in Fig. 1 (note: for the sake of simplicity, the curve for sublingual tablet is not illustrated, but because of the route of nicotine delivery, the plasma curve is qualitatively similar to nicotine gum).

2.1 Sustained Dosing Formulation: Transdermal Patch

Nicotine patches are applied to the skin, and deliver nicotine through the skin at a relatively steady rate. There are currently four patch formulations on the market in the USA and many other countries that vary in their design, pharmacokinetics, and duration of wear (i.e., 24- and 16-h wear). The diversity in patch systems has been described in reviews (Henningfield 1995; Gorsline 1993), and the differences in pharmacokinetics have been illustrated in a head-to-head clinical trial

(Fant et al. 2000). All of the patch types are available in a range of dosages, and progressively lower doses are used to provide weaning over a period of several weeks or longer, to enable gradual adjustment of the body to lower nicotine levels and, ultimately, to a nicotine-free state. Some formulations and indications also provide a lower dose for less dependent smokers.

Importantly, nicotine patches do not seem to provide protection against acute craving provoked by smoking-related stimuli. Tiffany et al. (2000) and Waters et al. (2004) showed that even though a nicotine patch reduced background craving compared to a placebo, smokers on active patches experienced similar boosts of craving when exposed to a provocative stimulus. Waters et al. also showed that wearing a nicotine patch had no effect on recovery from cue-provoked craving. As cue-provoked craving appears to be a major factor in relapse (Shiffman et al. 1996a), many authors have suggested supplementing patch wear with acute dosing formulations of NRT, which are described below.

2.2 Acute Dosing Formulations

There are several options available to smokers that, unlike the nicotine patch, allow them to self-administer a dose of nicotine on an "as needed" basis. These include nicotine gum, lozenge, sublingual tablet, oral inhaler, and nasal spray. All of these products, except the nasal spray, deliver nicotine through the oral mucosa. Acute-dosing products have the benefit that both the amount and timing of doses can be titrated by the user. Theoretically, therefore, smokers with more nicotine tolerance or greater need can get a higher nicotine dose, and smokers who are experiencing acute adverse effects can scale back their intake.

Control over the timing of self-dosing is key to the use of acute dosing products, because it enables smokers to use NRT as a "rescue medication" when they encounter particularly strong cravings or threats to abstinence. This form of use requires some explanation. Abstinence from tobacco causes some tonic disruptions of function, including rises in overall levels of craving that can be lowered by use of a transdermal patch. This background level of craving is punctuated, however, by acute episodes of more intense craving (Shiffman et al. 1997). These episodes of "breakthrough craving" are typically provoked by situational stimuli, such as seeing someone smoke or experiencing emotional upset (Sayette et al. 2000). These acute craving episodes are particularly problematic for smokers, and are associated with a very high risk of relapse (Shiffman et al. 1996b). Shiffman and coworkers demonstrated that one acute dosing product, nicotine gum, could reduce acute craving following exposure to a provocative stimulus (Shiffman et al. 2003). Some initial reductions in craving are likely due to the behavioral effects of medication use, such as the act of chewing the polacrilex gum (Cohen et al. 1997). Subsequently, however, the nicotine absorbed would itself begin to exert an effect and further reduce craving. Thus, smokers may be able to effectively use nicotine gum or other acute dosing products to enhance the relief provided by a transdermal patch and as a rescue medication when faced with acute threats to abstinence.

The first acute dose NRT that was made available to consumers was transmucosally delivered nicotine polacrilex ("nicotine gum"); the gum is available in two doses: 2 and 4 mg, delivering approximately 1 and 2 mg, respectively (Benowitz et al. 1987). In highly dependent smokers, the 4-mg is superior to the 2-mg gum (Tonnesen 1988; Herrera et al. 1995). About 50% of the nicotine in gum is absorbed (Benowitz et al. 1987). Since smokers take in approximately 1 mg of nicotine per cigarette (Benowitz and Jacob 1984), a smoker using 2-mg gum would have to use as many gums as cigarettes. A smoker using 4-mg gum would need to use 50% fewer pieces per day. Typical gum use, however, does not approach these levels (Curry et al. 2003), so most gum chewers do not match the daily nicotine levels achieved through cigarette smoking. Furthermore, because of the relatively slow absorption of nicotine from gum compared to smoke inhalation, individual doses do not produce the extremely high arterial levels of nicotine produced by smoke inhalation (Henningfield et al. 1993). Thus, nicotine gum is effective as a smoking cessation aid, while only partially replacing the patterns and levels of nicotine dosing obtained from cigarette smoking.

A 1-mg lozenge has been available in some European countries for some time; however, no efficacy data are available. A newer nicotine lozenge, available in 2- and 4-mg formulations has been approved in the USA, Europe, and Australia. Like nicotine gum, nicotine from the lozenge is absorbed gradually through the buccal mucosa and delivered into the systemic circulation. One advantage of the lozenge compared to gum is that chewing is not required. Also, the amount of nicotine absorbed per lozenge is somewhat higher than that delivered by gum, because all of the nicotine in the lozenge is delivered unless the lozenge is taken out of the mouth. In contrast, some amount of nicotine remains in the gum matrix even after 30 min of chewing. Single-dose studies have demonstrated 8–10% higher peak nicotine plasma concentrations and 25–27% higher total absorption of nicotine from lozenges compared to gums at both 2- and 4-mg dose levels, which is probably due to the residual nicotine retained in the gum (Choi et al. 2003).

A small sublingual nicotine tablet has been developed and is currently being marketed in many European countries, but is not yet available in the USA. The product is designed to be held under the tongue, where the nicotine is absorbed sublingually over about 30 min. The product that is currently available contains 2 mg nicotine, of which 1 mg is absorbed via the buccal mucosa. Compared to the gum and lozenge, the sublingual tablet demands even less activity from the user. The levels of nicotine obtained by use of the 2-mg tablet and 2-mg nicotine gum are similar (Molander and Lunell 2001).

The nicotine vapor inhaler consists of a mouthpiece and a plastic cartridge containing nicotine. When the inhaler is "puffed", nicotine is drawn through the mouthpiece into the mouth of the smoker. Each inhaler cartridge contains 10 mg nicotine, of which 4 mg can be delivered and 2 mg can be absorbed by use of a single inhaler (Molander et al. 1996). The product is not a lung inhaler, in that nicotine is not delivered to the bronchi or lungs, but rather deposited and absorbed in the mouth, much like nicotine gum (Bergstrom et al. 1995). The majority of nicotine is delivered into the oral cavity (36%) and to the esophagus and stomach (36%), with very

little nicotine reaching the lungs (4%). Nicotine delivery is related to the number and depth of inhalations. Labeling states that 80 deep puffs of the inhaler delivers 4 mg of nicotine; fewer or shallower puffs will deliver correspondingly smaller amounts of nicotine. Moreover, the amount of nicotine absorbed from the inhaler is temperature-dependent, with higher temperatures delivering larger amounts of nicotine and lower temperatures delivering smaller amounts (Lunell et al. 1997). One study observed very small plasma level increases produced by controlled puffing on the inhaler (Schuh et al. 1997). The vapor inhaler was designed to satisfy behavioral aspects of smoking, namely, the hand-to-mouth ritual. For some smokers, this may be a useful adjunct. However, this mechanism has not been directly tested.

Nicotine nasal spray is marketed as a pharmacy-only medication in the UK, and is available only by prescription in the USA. The nasal spray was designed to deliver doses of nicotine to the smoker more rapidly than other NRT products. The device is a multidose bottle with a pump that delivers 0.5 mg of nicotine per 50-μL squirt. Each dose consists of two squirts, one to each nostril. Nicotine from the nasal spray is absorbed into the blood more rapidly than from the gum (Schneider et al. 1996). Venous plasma concentrations after a single 1-mg dose range between 5 and 12 ng mL^{-1}. Time to peak plasma concentration (T_{max}) with nasal administration is around 11–13 min for 1-mg doses. This rise time is slower than for cigarette delivery (Henningfield et al. 1993), but faster than for the other NRT products.

A true pulmonary inhaler, unlike the currently available nicotine inhaler (which actually delivers nicotine into the mouth for buccal absorption), would deliver nicotine to the lung in a manner more comparable to cigarette smoking. This mode of delivery would be expected to reduce background cravings and withdrawal symptoms, and allow for rapid relief of acute cravings. In theory, because the delivery of nicotine directly to the lungs would more effectively mimic the effects of cigarette smoking on a physiological level, the smoker could more readily eliminate the need for tobacco, and subsequently taper the nicotine level over time to alleviate dependence upon nicotine altogether.

There are a number of challenges involved in the development of a pulmonary inhaler (Henningfield et al. 2000). Technical challenges are not trivial, as the nicotine molecules would need to be appropriately condensed onto particles of approximately 1 μm median diameter to enable inhalation into the pulmonary alveoli, and the nicotine particles must be designed so as to prevent the production of unacceptably harsh sensory effects and pulmonary pathology. Significant barriers to development of a pulmonary inhaler are the potential for abuse, and the regulatory implications that would follow from a system that delivers pulmonary nicotine at levels comparable to that delivered by a cigarette. Specifically, if the medication meets the criteria for a controlled substance, its marketing could be severely restricted along the lines of morphine-like analgesics. Such marketing restrictions can be expected to limit commercial development of such a product because of the uncertain market for a tobacco cessation product that is regulated as a controlled substance. This issue may require resolution by the WHO if the organization deems it important to encourage development of NRT products that deliver nicotine to the lung, or by other means that increase its abuse liability.

2.3 Combination Products

One strategy for further improving the efficacy of existing NRT medications is to combine one medication that allows for passive nicotine delivery (e.g., transdermal patch) with another medication that permits acute libitum nicotine delivery (e.g., gum, nasal spray, inhaler) (Sweeney et al. 2001). The rationale for combining NRT medications is that smokers may need both a slow delivery system to achieve a constant concentration of nicotine to relieve tonic cravings and tobacco withdrawal symptoms, as well as a faster-acting preparation to function as rescue medication for immediate relief from breakthrough cravings (Sweeney et al. 2001). Thus, combining the nicotine patch (which may prevent the appearance of severe withdrawal) with acute dosing forms (which can provide relief in trigger-to-smoke contexts) may provide an excellent treatment option over either therapy alone.

Clinical trials suggest incrementally increased efficacy of the patch plus gum, compared to either product alone (Fagerstrom et al. 1993; Kornitzer et al. 1995; Puska et al. 1995). Less research is available on combinations of the patch and other acute NRT formulations, but several studies suggest that combinations with other acute dosing forms also provide a clinical benefit, as would be expected (Blondal et al. 1999). Adding an acute dosing form to patch regimens yields substantial incremental benefit, whereas adding another patch (above) yields less benefit. This suggests that the mechanism is not simply an increase in nicotine dose, but the combination of steady-state dosing and acute dosing to provide for use as rescue medication. On the other hand, bupropion in combination with the nicotine patch appears to be more efficacious than the nicotine patch alone (Jorenby et al. 1999), possibly because the two medications act via different pharmacological mechanisms.

Despite the possibility of increased efficacy (Fiore et al. 2000), present NRT labeling warns against combination use. Without removal of such warnings, these strategies will be largely limited to smoking cessation specialists and clinics, or to the whims of consumers. The complexity of obtaining approval for combination medications, combined with the difficulty of marketing combination products, has slowed attempts by manufacturers to gain regulatory approval for combination therapies (Fiore et al. 2000).

2.4 Abuse Liability of Nicotine Replacement Medications

The addictiveness of a given substance goes beyond the chemical structure of the addictive drug itself (i.e., morphine, cocaine, or nicotine). The effects are also related to the dose and speed of delivery, as well as to other substances that might be part of the formulation. For example, just as the oral consumption of opioids and cocaine produce substantially less pronounced behavioral and physiological effects than intravenous or smoked consumption, slow release forms of nicotine produce generally less pronounced effects than smoked forms (Henningfield and Keenan 1993). Similarly, the “free base” or unprotonated forms of cocaine and

nicotine can produce more rapid absorption and stronger psychoactive effects when taken by mouth, and lung inhalation induces still more rapid effects (Cone 1995; Evans et al. 1996; Fant et al. 1999; Henningfield et al. 2004; Henningfield and Benowitz 2004). The amount of available nicotine per unit dose (e.g., single cigarette, piece of gum, or single patch) also varies substantially across products. Further, whereas medicinal nicotine products contain only nicotine as an active ingredient that contributes to its addictive effects, cigarettes likely contain a number of additional compounds that may contribute to addiction. This is consistent with the observations that 'tobacco-delivered nicotine' is more addictive and toxic than formulations provided by nicotine replacement medications (Royal College of Physicians 2000). For example, it has been suggested that MAO inhibitors present in tobacco smoke (Fowler et al. 1996a, b) may play a role in tobacco dependence. The potential of alkaloids and other substances in tobacco, in addition to nicotine, to which influence the overall psychopharmacological effects of tobacco use is also plausible, but has been little studied (Henningfield and Benowitz 2004).

As shown in Fig. 1, the speed of nicotine uptake in venous blood following several forms of nicotine delivery varies widely, from that of the very slow pattern of nicotine appearance in the blood (several hours to peak level) produced by current transdermal nicotine medications to the explosive rise produced by tobacco smoke inhalation. Nicotine gum, lozenge, tablet, and vapor inhaler can provide more rapid delivery of nicotine than the patch, but the speed and amount obtained are constrained by use patterns. Smokeless tobacco products deliver their nicotine more rapidly than nicotine gum and with less physical effort, but are still slower than cigarettes in their nicotine delivery.

Whereas the approximately 1–2 mg of nicotine delivered by smoking is highly reinforcing, the same dose delivered in the form of nicotine polacrilex gum provides a very low degree of reinforcement (Nemeth-Coslett and Henningfield 1986). Transdermal nicotine preparations deliver the drug at an overall rate of approximately $0.9\,\mathrm{mg\,h^{-1}}$, a rate that is virtually devoid of psychoactive effects, and provide little of the pleasure that cigarette smokers have come to expect of their nicotine delivering product (Pickworth et al. 1994). The more rapid nicotine delivery capability of a nasal nicotine preparation is consistent with its abuse liability profile, which appears to be substantially less than that of cigarettes, but greater than that observed with orally absorbed products (Schuh et al. 1997). An implication of these findings is that all nicotine medications may not be equally interchangeable or effective across patients. A corollary is that not all nicotine delivery systems warrant similar regulatory and marketing restrictions; those with higher risks of abuse and dependence are appropriately regulated and marketed more restrictively.

The long-term use patterns of various nicotine-containing products differ by dose and form. Clearly, dependent users of cigarettes and smokeless tobacco often use these products for years prior to making a quit attempt, and often take years to successfully quit. In contrast, users of medicinal nicotine tend to use the products for a much shorter duration. For example, one study found that among 805 households that purchased nicotine gum, 2.3% of new purchase incidents led to continuous monthly purchase of gum for $\geqslant$6 months. For nicotine patches (2050 households),

the percentage was 0.9%. For both gum and patch, the incidence of persistent purchase dropped below 0.4% by 24 months. The percentage of smokers who use nicotine nasal spray for a full year after cessation also appears low, even when subjects are instructed that they may use the spray for a full year (Blondal et al. 1999). These findings suggest that few people use medicinal nicotine products on a long-term basis, demonstrating a lower abuse potential than tobacco products.

3 Nicotine Partial Agonists

Nicotine acts at the nicotinic acetylcholine receptor (nAChR), which exists in a variety of subtypes, depending on the protein subunits from which they are comprised (Gotti et al. 2007). Most of the high-affinity binding is accounted for by receptors containing the alpha-4 and beta-2 subunits (Gotti et al. 2007). Nicotine is a full agonist at these receptors. Varenicline is a partial agonist (Mihalak et al. 2006). A partial agonist is a compound that, even at high doses, does not produce the same response as a full agonist. Because there is a ceiling on the effects of a partial agonist, it is plausible that a partial nicotine agonist would have a lower risk of adverse events and have a lower abuse potential than a medication containing nicotine. It is plausible that a compound that binds with a high degree of specificity or with a greater affinity to this subtype, relative to nicotine, will have a higher level of efficacy than nicotine itself. However, to the extent that other receptor subtypes might be associated with these effects, the efficacy could be muted compared to nicotine, which is less specific in its receptor affinity.

Phase III clinical trials showed significantly greater continuous abstinence rates in patients administered varenicline, compared to placebo or bupropion SR. Questionnaires administered during clinical trials assessed subjective feelings of the urge to smoke and nicotine withdrawal. Participants who had been administered varenicline had significantly lower scores for craving and withdrawal symptoms. Varenicline also showed an acceptable safety profile, with nausea, insomnia, abnormal dreams, and headaches being the most commonly reported adverse effects (Gonzales et al. 2006; Jorenby et al. 2006). Varenicline has been marketed in the UK, the USA, and several other countries since mid-2006, and has been subsequently approved in many other countries.

4 Nicotine Antagonists

Mecamylamine is a noncompetitive antagonist at the nicotinic acetylcholine receptor site. If mecamylamine could effectively block the physiological and reinforcing effects of cigarette smoking, this should, in theory, lead to eventual extinction of the behavior. When mecamylamine is administered to smokers, it has increased rather than decreased ad libitum smoking behavior, presumably due to smokers

compensating for partial receptor blockade, but it has also attenuated smoking satisfaction as well as other physiological, behavioral, and reinforcing effects of nicotine (Nemeth-Coslett et al. 1986). These effects are consistent with a partial pharmacological blockade. There is some evidence that mecamylamine may be useful for some recalcitrant smokers as a smoking cessation aid (Tennant et al. 1983). However, the side effects of the medication (hypotension, constipation) may limit its utility. Targacept is currently developing enantiomers of mecamylamine for the treatment of depression, which may have a more favorable safety profile than mecamylamine. It is unclear whether this enhanced safety profile, along with the theoretical utility in smoking cessation, will make these nicotine antagonists candidates for smoking cessation medications.

Mecamylamine in combination with nicotine transdermal medication has been investigated as a smoking cessation aid, and may produce better cessation outcomes than nicotine alone. One randomized, double-blind, placebo-controlled clinical trial found that a combination of the nicotine patch plus mecamylamine produced end-of-treatment abstinence rates three times higher than those for the nicotine patch alone, with benefits for the combined treatment group remaining apparent through 12 months (Rose et al. 1994). The addition of mecamylamine also significantly reduced cigarette craving, negative effects, and appetite. Side effects such as constipation and dizziness, however, were common. These results suggest that mecamylamine, combined with nicotine replacement, may ultimately prove to be a useful aid in smoking cessation. However, given that this study was conducted years ago and the product has not yet been marketed, it is possible that the combination did not warrant marketing, based upon low efficacy or a poor side-effect profile.

5 Nicotine Vaccines

A vaccine against nicotine induces antibodies that can bind nicotine molecules in plasma, theoretically before the drug reaches the neural receptors that produce effects normally associated with smoking. For example, in one study rats received either an active or a placebo vaccine, and 30 min later received nicotine at 0.03 mg kg^{-1} i.v., equivalent on a mg/kg basis to the nicotine intake from two cigarettes by a smoker (Pentel et al. 2000). Compared to control, the active vaccine reduced the brain nicotine concentration in a dose-related manner (65% reduction at the highest dose of vaccine). Pretreatment with the active vaccine also reduced the distribution to the brain of five repeated doses of nicotine (equivalent to the nicotine intake from ten cigarettes), administered over 80 min. Because vaccines reduce the amount of nicotine, and speed at which the nicotine reaches the brain and neural receptors, it would be predicted that the reinforcing effects of nicotine would be reduced substantially. This was supported in one study, which found that immunization with a nicotine vaccine prevented the nicotine-induced increase in dopamine release in the shell of the nucleus accumbens, a biochemical correlate to the rewarding properties of nicotine (de Villiers et al. 2002). Another study found that

exposure to nicotine after a period of extinction did not reinstate self-administration of nicotine among immunized rats, suggesting a muted reinforcing effect of nicotine (Lindblom et al. 2002).

Taken together, these results suggest that immunization using a nicotine vaccine could be used for smoking cessation. There are currently three companies in clinical development of an antinicotine vaccine: Cytos (Nicotine-Qbeta), Nabi (NicVAX), and Celtic (TA-NIC). Two companies are in preclinical development: Chilka and Independent Pharmaceutica (Niccine) (Siu and Tyndale 2007). Phase I trials have been completed for Nicotine-Qbeta, NicVAX, and Ta-NIC, showing each to be safe, well-tolerated, and capable of achieving nicotine-specific antibody responses (Hatsukami et al. 2005; Maurer et al. 2005; Xenova Group PLC 2005).

Cytos has successfully completed several phase II studies for Nicotine-Qbeta. The results from one study in 341 heavy smokers showed that a significant number of participants who achieved high antibody levels during administration of Nicotine-Qbeta met a criterion of continuous abstinence of smoking versus placebo. In participants who achieved low and medium antibody levels, there was no significant difference from placebo. Cytos is currently conducting a phase IIb/III optimized treatment regimen trial (Heading 2007).

Nabi has successfully completed several phase II studies for NicVAX. The results from a phase IIb dose-ranging study in 301 heavy smokers showed that a significantly greater number of vaccinated participants met abstinence endpoints compared to placebo. Furthermore, vaccinated participants who successfully achieved abstinence were shown to have a higher level of antibody response than those vaccinated participants who did not achieve abstinence (Nabi Biopharmaceuticals 2007). Nabi is expected to start phase III trials this year.

The potential mechanism and clinical utility of a nicotine vaccine is intriguing. In theory, by greatly reducing or eliminating the nicotine that reaches the brain, the reinforcing efficacy of tobacco smoking would also be reduced, eventually leading to extinction of the behavior (smoking). However, if the amount of nicotine that reaches the brain is reduced, rather than completely eliminated, it is possible that some smokers would actually increase tobacco consumption, at least in the short term, in order to achieve the levels of nicotine normally obtained during smoking. However, this sort of compensatory increase in nicotine consumption has not occurred so far in animal or human studies (Hatsukami et al. 2005; LeSage et al. 2003). Results of early research suggest that a nicotine vaccine would be useful as a relapse prevention treatment. The observation that animals did not reinstate nicotine self-administration after extinction when treated with vaccine (Lindblom et al. 2002) suggests that among people who quit smoking, a lapse (a single smoking bout) may not result in a full blown relapse because of the reduced reinforcing value of smoking. Vaccination typically produces long-lasting effects, so immunization might supplement the patients' willpower by eliminating the need for making daily or hourly decisions about smoking or taking antismoking medications. Finally, nicotine vaccines could theoretically be used in adolescents to prevent initiation of tobacco use. However, the risks, benefits, and ethical implications of such an intervention will undoubtedly require much more thorough evaluation before such application could be recommended (Hasman and Holm 2004).

6 Drugs Modulating Monoamine Neurotransmitters

As previously mentioned, many of the effects of nicotine in the brain are likely to be mediated through neuromodulation, in which nicotine potentiates the release of dopamine, norepinephrine, and serotonin (Picciotto 1998). By selectively activating these neurotransmitters, one might be able to mimic some of the reinforcing effects of nicotine.

Bupropion is an atypical antidepressant drug that is the only nonnicotine-based prescription medicine approved for smoking cessation by the FDA. Its mechanism of action is presumed to be mediated by its capacity to block neuronal reuptake of dopamine and/or norepinephrine (Fiore et al. 2000). Relative to other antidepressants, bupropion has a relatively high affinity for the dopamine transporter (Baldessarini 2001). There is also evidence that bupropion acts as a functional nicotine antagonist, suggesting another potential mechanism by which bupropion could reduce smoking rates (Slemmer et al. 2000).

Animal studies demonstrate that bupropion alters the reinforcing and withdrawal effects of nicotine. Low doses of bupropion reduce the rewarding effects of nicotine and the affective and somatic symptoms of withdrawal, as well as place aversion conditioned to nicotine withdrawal (Cryan et al. 2003; Malin et al. 2006). Another study examined the effects of bupropion (5–40 mg kg^{-1}) on the reinforcing properties of nicotine and food in rats, under two different schedules of reinforcement (Bruijnzeel and Markou 2003). The authors found that pretreatment with the highest dose of bupropion (40 mg kg^{-1}) resulted in a 50% reduction of nicotine intake in rats self-administering 0.03 mg kg^{-1} per infusion of nicotine under a fixed-ratio (FR) schedule. However, pretreatment with bupropion did not affect the self-administration of nicotine under a progressive-ratio (PR) schedule. These findings are challenging to interpret, but may indicate that a high dose of bupropion decreases the reinforcing properties of nicotine under conditions where doses can be obtained at regular and relatively short intervals, while leaving intact the motivation to work for nicotine when doses are more widely spaced. Taken together, these results suggest that bupropion has several actions demonstrated in animals that could explain its ability to increase rates of cessation in humans.

As a class, selective serotonin reuptake inhibitors (SSRIs) have a high affinity for the serotonin transporter, but a very low affinity for the dopamine transporter (Baldessarini 2001). On the whole, these medications have not shown promising effects as smoking cessation aids. For example, the selective serotonin reuptake inhibitor fluoxetine was shown not to be efficacious for smoking cessation (Niaura et al. 1995). Similarly, a clinical trial of venlafaxine showed no drug-placebo difference in abstinence rates at the end of a 10-week treatment trial (Cinciripini et al. 2004). However, two studies have shown that among abstinent smokers, there was less weight gain associated with cessation while using fluoxetine relative to placebo (Spring et al. 1995; Pomerleau et al. 1991). This suggests that SSRI medications might be useful for some smokers concerned about postcessation weight gain.

Tricyclic antidepressants have a relatively high affinity for both the serotonin and norepinephrine transporters, and some affinity for the dopamine transporter (Baldessarini 2001). Several clinical trials have demonstrated the potential efficacy of nortriptyline for smoking cessation in smokers without a history of major depression (Prochazka et al. 1998), or with such a history (Hall et al. 1998), and nortriptyline has been listed by the Agency for Health Research Quality as a second-line therapy (Fiore et al. 2000). A recent systematic meta-analysis of five randomized clinical trials suggests that the medication, because of the low cost, should be offered by physicians as a first-line therapy (Wagena et al. 2005). The tricyclic antidepressant doxepin has also been shown, in a small human study, to improve cessation rates (Edwards et al. 1989); however, larger studies are clearly needed to verify these findings. Other studies have shown that doxepin significantly reduces postcessation tobacco withdrawal symptoms and cigarette craving (Edwards et al. 1988; Murphy et al. 1990).

7 Dopamine D3 Receptor Antagonists

Dopamine D3 receptors (D3 DRs) are primarily localized in the shell of the nucleus accumbens, the ventral tegmental area, and the amygdala (Lévesque et al. 1992). Because of their increased expression in these brain regions that are central to reward, D3 DRs appear to play an important role in the mediation of the reinforcing effects of addictive drugs. Developmental antagonists that selectively block D3 DRs may have a potential therapeutic effect in modulating dopamine response to addictive drugs, including nicotine. One company, GlaxoSmithKline, is clinically evaluating a D3 DR antagonist that is currently in phase I trials.

Preclinical studies suggest that D3 DR antagonists reduce the influence of environmental stimuli associated with nicotine, but do not reduce response to aversive stimuli or natural reinforcers. For example, studies have shown that D3 DR antagonists reduced nicotine-conditioned place preference in rats, with no reduction in place preference to food (Le Foll et al. 2005; Pak et al. 2006) They also reduced nicotine-conditioned locomotor activity in rats (Le Foll et al. 2003; Pak et al. 2006). These results may be especially relevant to smoking cessation, because conditioning processes appear to play a large role in smoking behavior.

D3 DR antagonists also appear to reduce nicotine-relapse behavior and self-administration at higher doses. One study found that a relatively low dose (3–10 mg kg^{-1}) of a D3 DR antagonist reduced the reinstatement of nicotine self-administration behavior, but not self-administration per se (Andreoli et al. 2003). However, a recent study showed that a higher dose (56 mg kg^{-1}) significantly reduced nicotine self-administration response rates, but not food self-administration (Ross et al. 2007). Furthermore, Pak et al. (2006) showed that a D3 DR antagonist prevented nicotine-potentiated brain stimulation reward, suggesting a reduction in the rewarding effects of nicotine.

8 α_2-Noradrenergic Agonists

Clonidine is an α_2-noradrenergic agonist used in the treatment of hypertension. Clonidine has been shown to diminish symptoms of both opioid and alcohol withdrawal (Gossop 1988; Mayo-Smith 1998). On the other hand, the Clinical Practice Guidelines have given clonidine a B level of evidence, indicating that there is some evidence of efficacy (Fiore et al. 2000). For example, one study of heavy smokers who had failed in previous quit attempts found, at the end of the 4-week treatment, that those treated with clonidine had twice the rate of abstinence as those treated with a placebo (Glassman et al. 1988). This effect continued through the 6-month follow-up. These results suggest that clonidine may be efficacious in the treatment of tobacco dependence, but the conditions under which it is most appropriately used are not well defined.

The most common side effects of clonidine are constipation, dizziness, drowsiness, dryness of mouth, and unusual tiredness or weakness. However, there are more severe side effects that clinicians and patients should be aware of, such as allergic reaction, decreased heart rate, or unusually elevated or decreased blood pressure, as well as contraindications and drug interactions that should be evaluated prior to prescription.

9 Cannabinoid Antagonists

The cannabinoid-1 (CB1) receptor plays a role in the regulation of appetitive behavior. For example, Black (2004) found that exogenously administered cannabinoid receptor agonists stimulate food consumption in animals and humans. The endocannabinoid system also appears to mediate the effects of nicotine in the brain. Cohen et al. (2002) evaluated the effects of rimonabant, a CB1 receptor antagonist, on the motivational effects of nicotine in the rat. Administration of rimonabant (0.3 and 1 mg kg^{-1}) decreased nicotine self-administration (0.03 mg kg^{-1} per injection). Rimonabant (0.3–3 mg kg^{-1}) neither substituted for nor antagonized the nicotine cue in a nicotine discrimination procedure. Secondly, using brain microdialysis, rimonabant (1–3 mg kg^{-1}) blocked nicotine-induced dopamine release in the shell of the nucleus accumbens (NAc) and the bed nucleus of the stria terminalis. These results suggest that activation of the endogenous cannabinoid system may participate in the motivational and dopamine-releasing effects of nicotine.

Of the three studies of rimonabant for smoking cessation, STRATUS-US is the first to be completed, and the findings of this study were presented at the 2004 American College of Cardiology annual meeting (Cleland et al. 2004). The study found that a 20-mg dose doubled abstinence rates, compared to placebo and a 5-mg dose. Importantly, smokers who quit in the 20-mg group gained less weight than those that quit in the placebo group. Weight gain is a common side effect of smoking cessation, with the average gain being as much as 13 pounds after one year of continuous abstinence (Klesges et al. 1997). Furthermore, many smokers report weight

gain to be one of the factors associated with relapse (Klesges et al. 1989). Thus a medication that reduces the weight gain associated with cessation may decrease the likelihood of relapse during a quit attempt. Relative to placebo, the most commonly elevated side effects of 20 mg rimonabant were nausea and upper respiratory tract infection. No cardiovascular safety concerns were identified with rimonabant.

STRATUS-WORLDWIDE was the second study of smoking cessation to be completed. The study examined the one-year treatment outcomes among smokers in the STRATUS-US trial who were abstinent after 10 weeks. The participants were further randomized to receive placebo, 5 mg of rimonabant, or 20 mg of rimonabant for an additional 42 weeks. Subjects who had been abstinent at 10 weeks on 20-mg were randomized into placebo, or rimonabant 5- or 20-mg groups. Subjects who had been abstinent at 10 weeks on 5 mg of rimonabant were randomized into placebo or rimonabant 5-mg groups. Significantly higher abstinence was achieved after 52 weeks by patients who were initially treated with 20-mg rimonabant and randomized to the 5- or 20-mg groups. In addition, those who received 20 mg throughout the treatment had significantly lower postcessation weight gain than placebo. In both studies, the most common side effects of rimonabant were nausea and upper respiratory tract infection. No cardiovascular safety concerns were identified with rimonabant (Steinberg and Foulds 2007).

Rimonabant presents advantages in preventing postcessation weight gain, which is viewed by many smokers as an adverse effect of quitting, as well as in providing a positive cardiovascular profile, in contrast to NRT. However, increased psychiatric side effects that appeared during clinical trials may prevent regulatory barriers. For example, the Rimonabant In Obesity (RIO) North America trials revealed increased rates of depressed mood and anxiety among the 20-mg rimonabant group versus placebo (Pi-Sunyer et at. 2006).

10 Discussion

It is now clear that treatment with smoking cessation medications is an efficacious and cost-effective path to disease control and prevention of premature mortality. There is a strong medical and public health need for pharmacotherapies to aid smokers who wish to quit smoking, but who are unable to do so without such assistance (World Health Organization 2003; World Bank 1999; Fiore et al. 2000; Royal College of Physicians of London 2000). It is equally clear, however, that many people find currently available treatments ineffective or unacceptable. Thus, the benefits and limitations of presently available treatments provide a powerful impetus for further treatment development.

Nicotine replacement medications have been used for over two decades to help smokers quit. However, nicotine delivery from medications could potentially be improved by formulations that better mimic the effects of tobacco-delivered nicotine. Antidepressants such as bupropion have also been shown to aid smoking cessation. Bupropion was initially studied for smoking cessation based upon anecdotal reports

from patients using the medication for the treatment of depression. Subsequent animal studies have demonstrated effects such as decreases in nicotine reinforcement and in withdrawal symptoms, suggesting potential mechanisms contributing to bupropion's efficacy. However, additional development of medications that act to control negative moods may be beneficial for smokers trying to quit.

In addition to these strategies, there are a variety of novel clinical and pharmacological targets being considered for future drug development. These include medications that treat withdrawal symptoms other than depression, novel partial agonists, and medications that target neurotransmitter systems other than the nicotinic acetylcholine receptor. The high cost and public health impact of smoking, along with the demonstrated interest of smokers in quitting, is fueling medication development efforts that will alter the future landscape of cessation aids available to the public.

New medications may provide alternatives to current treatments with similar average efficacy, or may actually prove effective in people who are refractory to presently available treatments. The degree to which new medications are acceptable and efficacious in new populations will be an important determinant of their ultimate contribution to public health. It is also possible that currently available medications could be used in new ways to reduce the long-term disease risk of smoking.

For example, medications might enable lasting smoking reduction in persons unable or unwilling to completely give up tobacco, thus reducing disease risk. Alternatively, by enabling short-term abstinence through the treatment of withdrawal, medications may prove to be an important gateway to eventual complete cessation. These and other medication options have enormous promise to contribute to global health in the face of the projected one billion premature tobacco-caused deaths that could occur in the twenty-first century, based on current trends (World Health Organization 2003).

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Nicotine Psychopharmacology: Policy and Regulatory

Jack E. Henningfield and Mitch Zeller

Contents

Abstract Powerful nerve agent, poison, addictive drug, or wonder medicine of the future? Nicotine has had a long and storied history in pharmacology, physiology, public health and, more recently, in regulatory policy initiatives in the United States and internationally. Psychopharmacology research on nicotine and tobacco came to particular prominence in the latter third of the twentieth century with exploration addressing the role of nicotine in tobacco use, the potential categorization of nicotine as an addictive drug, the pharmacological basis for treatment of tobacco addiction, and the perspective of policy developers seeking to reduce the toll of tobacco use. In fact, the 2005 ratification of the World Health Organization's first global health treaty, the Framework Convention on Tobacco Control, provides further impetus for extending the science foundation for tobacco disease control and policy efforts.

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Implementation of the treaty's provisions will control tobacco use and reduce the 500 million premature deaths projected to occur in the first half of the twenty-first century from tobacco use. Psychopharmacological research on nicotine and tobacco was important in the rationale and development of the treaty. The public health relevance of psychopharmacology research continues to grow with the realization of the potential of nicotine and related drugs to treat or prevent a diverse range of disorders (e.g., Alzheimer's disease, attention deficit hyperactivity disorder, and pain). Although comprehensive review of the research and implications is beyond the scope of this article, the more modest goal of providing insight into the theoretical, clinical, and policy importance of key psychopharmacology research laboratories over the past few decades is attempted.

On February 27, 2005, an International Treaty, the Framework Convention on Tobacco Control, entered into force with the recognition that tobacco products are addictive and with encouragement for further research to guide implementation of the treaty. Specifically, the treaty recognized that

> cigarettes and some other products containing tobacco are highly engineered so as to create and maintain dependence, and that many of the compounds they contain and the smoke they produce are pharmacologically active, toxic, mutagenic and carcinogenic, and that tobacco dependence is separately classified as a disorder in major international classifications of diseases.

Among the thousands of chemically distinct constituents of tobacco and smoke, many are highly toxic with disease and premature death resulting from repeated exposure. Nicotine is unique in that its direct toxicity at doses typically ingested is relatively low. Rather, its main contribution to disease is to drive the behavior of persistent tobacco use and toxicant exposure by its addicting actions. This pharmacologically potent and powerful molecule has diverse effects that drive voracious tobacco consumption by more than 1.3 billion people world-wide, despite the desire of many of them to cease consumption. The effects of nicotine on various organ systems are diverse and include the ability to stimulate heart rate, modulate various hormones, produce nausea, and relax skeletal muscle, depending upon the dose and speed of administration. These actions are probably not the primary driving forces in tobacco consumption, however. Rather, it is the psychopharmacological effects of nicotine, including discrimination, reinforcement, alteration of mood and feeling, and modulation of cognition, that are prominent determinants of the initiation, maintenance, and relapse into tobacco use.

Earlier, the World Bank had advocated increasing international efforts to control tobacco in view of the devastating impact of tobacco disease on developing nations and in the light of the "addictive nature of tobacco smoking" (World Bank 1999). At the national level, many nations are beginning to develop regulations for tobacco that are modeled in many respects after drug regulations but without the goal of necessarily banning the products – in part due to concerns that the psychopharmacology of tobacco dependence and withdrawal is so powerful that banning the products (at least in the near term) might itself precipitate health and social problems (Food and Drug Administration 1995, 1996; Henningfield et al. 1998). What is it about the

psychopharmacology of this seemingly simple alkaloid that has led to such efforts to contain its use and effects?

Psychopharmacology research helped build the foundation for the international regulation of tobacco (Henningfield and Zeller 2006). It was recognized that the psychopharmacological effects of nicotine, and perhaps other substances, led to tobacco addiction, which in turn generated the decades of daily exposure that so frequently results in debilitating disease and premature death. Further, it was learned that the tobacco industry itself had studied the psychopharmacology of nicotine for decades before acknowledging, in about 2000, that the psychopharmacology of nicotine was critical to tobacco use. The industry used this knowledge to foster use and addiction to its products (World Health Organization 2001). Much of this research was kept secret (World Health Organization 2001; Hurt and Robertson 1998; Wayne et al. 2004, 2006, 2008).

Fortunately, in the late 1970s, governments and nontobacco industry organizations began to greatly expand their support of psychopharmacology-related research on tobacco and nicotine. This led to increasing understanding of the contribution of nicotine to tobacco use and dependence (USDHHS 1988; Henningfield and Goldberg 1988; Food and Drug Administration 1995, 1996; Royal College of Physicians 2000; Henningfield et al. 2006; Miczek et al. 2006). The focus of this article is on nicotine from the perspective of psychopharmacological research and the implications of that research for public health, regulatory policy, and emerging research challenges.

1 The Dawn of Neuropharmacology and the Science of Drug Addiction

Nicotine was first isolated from tobacco by Posselt and Reimann in 1828 (US Department of Health and Human Services 1988). They concluded that it was a dangerous poison, recognizing its high degree of potency, powerful physiological effects and potential application as a pesticide (Domino 1999). Physiologists and pharmacologists in the early and mid twentieth century used nicotine as a tool to explore the nervous system (Domino 1999; US Department of Health and Human Services 1988). By the end of the nineteenth century, nicotine was increasingly used in studies of the peripheral nervous system. In fact, Langley and colleagues used nicotine to essentially map what became known as the "nicotinic" cholinergic peripheral nervous system (Langley 1905).

Following his extensive studies of nicotine, curare, and other chemicals acting on what is now known as the cholinergic nervous system, John Langley hypothesized that there must be "receptive substances" present in cells to explain the effects of nicotine alone and in combination with other chemicals. He wrote: "Since the accessory substance is the recipient of stimuli which it transfers to the contractile material, we may speak of it as the *receptive substance* (italics in original) of the muscle" (Langley 1905). These conclusions followed from observations

including the following: applied to the same muscle tissue, nicotine could activate, curare could abolish, and the drugs applied simultaneously might have no effect depending upon the amounts applied (Langley 1905). Langley demonstrated complex dose–response relationships whereby nicotine could produce stronger muscular reactions as the dose increased until a point was reached at which paralysis emerged. He explored antagonism of curare-induced muscle paralysis by nicotine, the development of tolerance to repeated nicotine dosing, and the recovery of responsiveness following several hours or more in which nicotine was not administered. The rich history of this pioneering research is beyond the scope of the present article, but this work was the foundation for work continuing into the twenty-first century.

Following early twentieth century exploration of the effects of tobacco and nicotine on physiology, mood and "psychic" functioning, the physiologist Louis Lewin drew seminal conclusions regarding nicotine in his treatise: "Phantastica, narcotic and stimulating drugs, their use and abuse," which was written in 1924 and reprinted extensively, including the 1998 version cited for this article (Lewin 1998). The following conclusions remain the cornerstone of much of today's research and policy development:

> In my own view cigarette smoking is the most dangerous manner of utilizing tobacco. The decisive factor in the effects of tobacco, desired or undesired, is nicotine, which is contained to the extent of from 2 to over 7 percent, according to the kind of tobacco and it matters little whether it passes directly into the organism or whether it is smoked

(Lewin 1998). Lewin described the various abilities of tobacco to intoxicate at high doses, as well as ritualistic and even presumed medicinal uses, attributing many, though not all, of the effects to the nicotine.

As the harmful effects of tobacco were uncertain, and the presumption was that many tobacco users benefited by its presumed abilities to enhance concentration and attention, tobacco was not generally categorized along with drugs such as opioids, alcohol, and marijuana, which were known to be capable of producing severe behavioral intoxication and more broadly accepted harmful effects, at least in heavy users. Nonetheless, the tobacco industry itself began major research on the role of nicotine in smoking in the midtwentieth century and by at least the early 1960s, it came to the conclusion that nicotine was "the sine qua non of smoking" and addictive. (Hurt and Robertson 1998; World Health Organization 2001; Henningfield 2004; Slade et al. 1995).

2 1964 Surgeon General's Report on Smoking and Health

The landmark 1964 Surgeon General's report on Smoking and Health came to several conclusions, but two are of particular relevance to the present analysis. The first was that cigarette smoking is a cause of lung cancer and elevated risk of death. The second was that cigarette smoking was appropriately categorized as a "habituation" and not an "addiction." The first conclusion irrefutably established the serious harm of cigarette smoking and laid the foundation for actions of the federal government

to take measures to reduce tobacco use and tobacco-caused disease. Although we now know that the tobacco industry itself understood that nicotine was addicting, it did not make its data and insights available to the Advisory Committee to the Surgeon General (Henningfield 2004; Hurt and Robertson 1998; Slade et al. 1995). This fact and the reliance of the Advisory Committee on a definition of addiction that was abandoned in 2004, contributed to the finding of "habituation" and not "addiction." The second conclusion may well have delayed regulatory and legal action built around the cornerstone principle that the nicotine in tobacco products is addictive (Henningfield 2004; Kessler 2001).

Interestingly, this report did take into account two important earlier studies that now would have fallen into the domain of psychopharmacology research. Neither of the studies had been replicated or extended so as to provide more definitive results regarding the addiction question. The first, by Johnston (1941), demonstrated that nicotine injections produce distinct psychoactive effects and could provide a substitute for cigarette smoke, supporting the conclusion that "smoking tobacco is essentially a means of administering nicotine, just as smoking opium is a means of administering morphine" (Johnston and Glasg 1941). The second study, by Finnegan, Larson and Haag (1945) investigated the effects of switching volunteers from cigarettes with regular nicotine levels to cigarettes with low concentrations of nicotine (Finnegan et al. 1945). Many smokers experienced discomfort, irritability, decreased ability to concentrate, and a feeling of "inner hunger" as though they had stopped smoking.

It wasn't until the 1980s that researchers systematically replicated these studies. The 1980s studies demonstrated unequivocally that nicotine was a potent and powerful psychoactive drug and that tobacco withdrawal was pharmacologically mediated by nicotine deprivation and modulated by environmental factors (Henningfield et al. 1985; Hughes and Hatsukami 1986).

3 Explosion of Nicotine Psychopharmacology Research in the 1970s

In 1967, Lucchesi, Schuster, and Emley demonstrated that nicotine infusions could reduce smoking, paving the way for the development of nicotine gum as a replacement therapy for tobacco dependence (Ferno 1977, 1973). This development provided both conceptual stimulus and a practical pharmacological tool for further exploration of nicotine in tobacco users. Many psychopharmacological researchers turned their focus to nicotine in the 1970s. This research laid the groundwork for the conclusions reached a decade later by the American Psychiatric Association, the US National Institute on Drug Abuse, the US Surgeon General, and many other domestic and global organizations, that tobacco was highly addictive and that nicotine was the addictive drug in tobacco that defined the addiction and exerted considerable control over tobacco use patterns (National Institute on Drug Abuse 1984; Food and Drug Administration 1996). This powerful body of work has been reviewed

elsewhere but some key findings need to be summarized to provide a context for policy development, regulation and twenty-first century challenges (US Department of Health and Human Services, 1988; Royal College of Physicians 2000).

The critical nicotine work in the 1970s was done by several psychopharmacologists who specialized in understanding the discriminative effects of the drug in animals (often referred to as "psychoactive" or "mood-altering" in humans). Some of their key findings were presented at the American Psychological Association (Henningfield and Hartel 1999) and published in this journal. They built on preliminary work by Donald Overton (1969) and Johns Rosecrans et al. (1978) whose studies had demonstrated that nicotine produced dose–response effects on behavior that were more similar to stimulants than sedatives, but still unique (Overton 1969; Rosecrans et al. 1978). Rosecrans' work included challenges with centrally and peripherally acting antagonists, leading to the conclusion that it was the effects of nicotine in the brain and not the peripheral nervous system that were critical determinants of nicotine's psychoactivity.

Another prominent psychopharmacologist, Murray Jarvik, cultivated some of the most prolific researchers and produced studies over several decades that helped to categorize nicotine as "addictive" or "dependence producing." Following his mixed success at establishing rhesus monkey models of cigarette smoking, Jarvik became convinced that an understanding of nicotine's role in smoking could be achieved by unraveling the interactions between nicotine dose and behavior (Jarvik 1977). Unfortunately, one of the most fundamental of pharmacological tools was not available, namely, cigarettes that were identical in sensory and pharmacological characteristics to conventional tobacco cigarettes but devoid of the target drug, viz. nicotine. Nonetheless, several highly fruitful lines of research explored the nicotine hypothesis. Ian Stolerman and colleagues demonstrated that cigarette consumption increases when the CNS effects of nicotine were blocked by the centrally and peripherally acting ganglionic blocker, mecamylamine, but not by the noncentrally acting ganglionic blocker, pentolinium (Stolerman et al. 1973). Taking a somewhat different tact, Saul Shiffman focused his efforts on quantifying tobacco craving and withdrawal. This led to one of the earliest widely adopted scales for assessing withdrawal: the Shiffman Jarvik Tobacco Withdrawal scale (Shiffman and Jarvik 1976).

Another monumental series of studies was emerging concurrently in Europe in the laboratories led by, and collaborating with, Michael Russell in London. Although not as focused on psychopharmacology, the work of Russell and colleagues contributed heavily to understanding the psychopharmacology of nicotine, its interaction with nicotine pharmacokinetics, and the value of nicotine medications for the treatment of tobacco dependence and withdrawal. The effort began with a theoretical analysis by Russell of the plausibility of the hypothesis that nicotine should be considered an addictive drug and of the extensive range of research questions that needed answers to come to a definitive resolution of this hypothesis (Russell 1971). The work of the Russell group confirmed that nicotine dose was a critical determinant of the effects of tobacco and of smoke intake, and that there was an orderly, albeit complex, relationship between nicotine blood levels and cigarette smoking. Russell's group also explored the advantages and disadvantages of many potential

routes of nicotine administration for controlling smoking, and to be used as aids to cessation (Russell 1988).

By the late 1970s, Russell was convinced that cigarette smoking was appropriately categorized as a form of drug addiction. Nevertheless, apparent inconsistencies in some of the lines of evidence led Russell to question whether nicotine was "rewarding" or "aversive" (Russell 1979). In particular, he pointed out (i) that the relationship between nicotine yield of cigarettes and smoking was very crude, (ii) that reduced smoking ("downward titration") in response to high nicotine-yielding cigarettes occurred more reliably than increased smoking in response to low nicotine yielding cigarettes, and (iii) that an animal model of nicotine self-administration similar to those existing for protoypic drugs of abuse did not exist to demonstrate unequivocally that nicotine could serve as a positive reinforcer (see also reviews by Gritz 1980; Griffiths et al. 1980; Henningfield 1984).

During the 1970s as well, tobacco cessation as an area of research and clinical practice was dramatically increasing, no doubt as a result of the 1964 Surgeon General's report and heightened education about the medical importance of tobacco cessation (US Department of Health, Education, and Welfare 1979; Schwartz 1987). Prominent addiction researchers and clinicians came to the conclusion that tobacco use can be a true drug addiction or "dependence." (Jaffe and Jarvik 1978). Despite gaps in knowledge concerning the psychopharmacology of nicotine, the evidence that many cigarette smokers met criteria for drug dependence was sufficient that the third revision of the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-III) included tobacco dependence along with five other classes of dependence-producing substances (e.g., alcohol, barbiturates, and opioids) (American Psychiatric Association 1980). The DSM-III also listed tobacco withdrawal among its "organic brain syndromes" drawing heavily on the Shiffman Jarvik scale for its symptom listing and description.

Since about the 1970s, psychopharmacologists have increasingly studied nicotine as a fascinating behaviorally active drug in its own right, as well as the apparent key to understanding tobacco use and addiction. Molecular pharmacologists and pharmaceutical developers, postulating great possibilities for nicotine analogs, devoted considerable attention to nicotine, beginning in about the 1980s. It is plausible that nicotine and its molecular analogs may have medicinal application in a wide variety of diseases including Parkinson's, Alzheimer's, Crohn's, attention deficit hyperactivity disorders, various cognitive disorders, and for the relief of pain (Balfour and Fagerstrom 1996). This research and still other lines of pychopharmacology research are also contributing to new models for treatment development (Lerman et al. 2007).

4 Scientific Questions Concerning Nicotine as an Addictive Drug

During the late 1970s, the National Institute on Drug Abuse (NIDA) held a series of scientific conferences that produced monographs focusing strongly on the extant knowledge on nicotine psychopharmacology, the state of the science, and the key

questions in need of resolution. This opened the door to more extensive support of tobacco and nicotine research by NIDA (Krasnegor 1979a, b). This groundbreaking work was inspired by the above-described research, the growing recognition of the enormous toll of tobacco use, and the limited progress in controlling smoking since the 1964 Surgeon General's report.

Existing data in the 1970s suggested that nicotine had the hallmark properties of an addictive drug. It was concluded that turning the attention of experienced drug addiction researchers to nicotine and tobacco had the potential to resolve key issues (Jarvik et al. 1977; Krasnegor 1979a, b; Pinney 1979). The NIDA conferences led to expanded funding of tobacco and nicotine research by the agency. Several psychopharmacology-focused laboratories and programs, which had already begun nicotine research in the 1970s, submitted grant proposals, were funded, and contributed to advances in psychopharmacology.

The laboratory most directly influenced by these conferences was undoubtedly NIDA's own Intramural Research Program ("Addiction Research Center"). In 1979 and 1980, the program recruited Steven Goldberg and Jack Henningfield to study the abuse liability of nicotine using standard animal and human models (Henningfield and Hartel 1999). Their work included the first demonstration that nicotine was self-administered at high rates by nonhuman primates (Goldberg et al. 1981, 1983), that humans would self-administer intravenous nicotine (Henningfield et al. 1983; Goldberg et al. 1983), and that intravenous nicotine produced psychoactive effects characteristic of prototypic controlled substances (Henningfield et al. 1985). This body of work enabled the then NIDA Director William Pollin to testify before Congress in 1982 and 1983 that nicotine met all the criteria of a dependence-producing drug (National Institute on Drug Abuse 1984; US Department of Health and Human Services 1984; Henningfield 2004).

5 1980s Research Strengthened the Foundation for the 1988 Surgeon General's Report and FDA Regulation of Tobacco

When NIDA enlarged its tobacco and nicotine research portfolio many psychopharmacology researchers were poised for this endeavor. They found the new research questions challenging, the opportunity to contribute to public health great, and they were eager to extend the experience and knowledge gained from the study of other psychoactive substances to this one. Key venues for intellectual exchange included the annual meetings of the American Psychological Association's Psychopharmacology Division (Henningfield and Goldberg 1983) and the College on Problems of Drug Dependence (Harris 1988). The application of what was, by then, a maturing scientific field of behavioral pharmacology, to the study of tobacco use proved efficient and productive. The scientific stream of information quickly turned into a river that fed developments in treatment, prevention, policy and, ultimately, regulatory efforts by the FDA, WHO and other national and international organizations (Food and Drug Administration 1995, 1996).

One especially productive program was that of the University of California, San Francisco which included Neal Benowitz, Sharon Hall, Ronald Herning, Peyton Jacobs and Reese Jones. This group of investigators explored the interaction between cigarette smoking and plasma nicotine levels, the variation in human smoking in response to cigarette variation and nicotine administration, and pharmacotherapy (Hall et al. 1987; Benowitz et al. 1983). Particularly important was research that indicated that the Federal Trade Commission (FTC) ratings of cigarettes were virtually meaningless predictors of smoke and nicotine intake. This helped explain why earlier studies of nicotine intake, in which cigarettes of differing FTC yields were used to vary dose, had often yielded seemingly small changes in behavior. The work of Benowitz et al. suggested that the intended dose manipulations had been virtually meaningless in many studies because relatively subtle changes in smoking pattern could sustain the desired intake regardless of the rating of the cigarette (Benowitz et al. 1983; National Cancer Institute 1996).

The Jarvik laboratory at UCLA was also quite active during the 1980s (Gritz 1980) exploring nicotine intake regulation by empirical research and analysis. Nina Schneider explored the control of smoking by nicotine and its potential treatment by administering the then-experimental nicotine gum (Schneider et al. 1984). These researchers laid the foundation for the work of another Jarvik protégé, Jed Rose. In the early 1980s, Rose explored the possibility that nicotine absorbed through the skin might reduce urges to smoke, leading to the first published "nicotine patch" studies (Rose et al. 1984).

Another psychopharmacology program was at Johns Hopkins University School of Medicine and led by Roland Griffiths, George Bigelow, Maxine Stitzer, and Joseph Brady. This program examined cigarette smoking from a classic behavioral pharmacological perspective, varying dose, cost, access, and deprivation state, as well as many drug interaction studies. They demonstrated that cigarette smoking indeed resembled a prototypic psychoactive substance of abuse along key dimensions, including the patterns of smoke intake in response to manipulations of smoke dose, access, response cost, and deprivation state (Griffiths and Henningfield 1982; Henningfield and Hartel 1999). The drug interaction studies were particularly interesting in that most drugs that produced euphoria increased subsequent cigarette smoking with the exception of nicotine itself, for which nicotine supplementation resulted in decreased smoking (Henningfield 1984).

Taken together, the psychopharmacology research of the 1970s and 1980s provided a strong foundation for the conclusions by the National Institute on Drug Abuse (National Institute on Drug Abuse 1984) and the Surgeon General (National Institute on Drug Abuse 1987) that nicotine was addicting. In turn, this research and the reports of the National Institute on Drug Abuse provided support to the 1988 Surgeon General's report (US Department of Health and Human Services 1988), which came to the following three main conclusions:

1. Cigarettes and other forms of tobacco are addicting
2. Nicotine is the drug in tobacco that causes addiction
3. The pharmacologic and behavioral processes that determine tobacco addiction are similar to those that determine addiction to drugs such as heroin and cocaine

6 Nicotine Psychopharmacology Research was Pivotal in FDA's Conclusions that Cigarettes and Smokeless Tobacco are Addicting

There are many other laboratories and studies that space does not permit mentioning in this brief survey. However, a few were particularly relevant to FDA's subsequent deliberations (described below) concerning tobacco/nicotine regulatory policy. The University of Minnesota team led by John Hughes, Dorothy Hatsukami, and Roy Pickens developed a program that extensively evaluated the nature and extent of tobacco withdrawal in humans (Hatsukami et al. 1984; Hughes and Hatsukami 1986). This work provided a strong scientific foundation for FDA's conclusions regarding the prominence of addiction in the use of tobacco. For example, Alan Collins and his colleagues explored the relationship between brain nicotine receptors, behavior, and tolerance (Collins et al. 1988; Marks et al. 1986). This work complemented other research demonstrating the effects of nicotine on nicotine receptor density, brain energy utilization, and hormone regulation. All of this work supported the FDA's conclusions that the addictive actions of tobacco were caused in part by the effects of nicotine on the structure and function of the body (Schwartz and Kellar 1983; Henningfield et al. 1987, 1996; London et al. 1988; Grunberg et al. 1988).

Early 1990s research on nicotine discrimination (Perkins et al. 1994a, b), self-administration (Corrigall 1999), and the role of brain nicotine receptors in behavior and withdrawal (Markou 2008) lent further support to the conclusion that nicotine was appropriately categorized as an addictive drug and buttressed FDA's scientific basis for asserting jurisdiction over nicotine-containing tobacco products (Food and Drug Administration 1995, 1996).

The research described above, coupled with the conclusions of the 1988 Surgeon General's report, enabled the FDA Commissioner David Kessler to testify before the US Congress on 25 March 1994 that nicotine was addictive and that the FDA would investigate the possibility that the agency should regulate tobacco as a drug. In his testimony, Kessler discussed nicotine self-administration, its psychoactive effects, the behavioral effects of nicotine including modulation of cigarette smoke intake, and the importance of brain mechanisms of nicotine action (Food and Drug Administration 1995; Kessler 1995). He also testified that the tobacco industry itself conducted pivotal research on the addictive effects of nicotine and that some of the findings were suppressed. As part of that testimony, Jack Henningfield (1995) summarized the results and implications of the research of Victor DeNoble and Paul Mele at Philip Morris. The suppression of these findings and the termination of this research have been concluded to have held back the development of treatments for tobacco dependence and other efforts that could have accelerated public health advancement, in addition to representing an unfortunate instance of censorship of scientific communication (Henningfield 1995, 2004; Barry 2005; Food and Drug Administration 1996; Kessler 2001).

On 23 August, 1995, at a White House ceremony, the FDA released its preliminary determination to regulate cigarettes and smokeless tobacco as combination drug/device products. This action gave the agency the flexibility to regulate them without banning them. The contributions of the overwhelming psychopharmacological evidence for FDA's bedrock scientific conclusions cannot be overstated. Of course, epidemiological data on the nature and extent of tobacco use, addiction, and morbidity were important, as were tobacco industry documents confirming the industry's knowledge and intent to design and manufacture cigarettes as though they were drug delivery devices with the intent of causing and sustaining dependence (Food and Drug Administration 1995). Following approximately 6 months of public commentary and the receipt of more than 700,000 comments, the FDA issued its assertion of jurisdiction and final rule in August 1996 (Food and Drug Administration 1996).

Key actions authorized by the rule were aimed at reducing access to tobacco by children and adolescents and reducing the appeal of tobacco products to young people by restricting marketing and advertising practices. The FDA acknowledged problems with labeling (e.g., "light" and "low tar"), the FTC system of measuring tar and nicotine levels (National Cancer Institute 1996), and the need to develop a science base for regulating ingredients and design (Food and Drug Administration 1996). The agency noted that addressing such issues required time to develop the science base and infrastructure for such regulation (Kessler 2001).

The tobacco industry immediately filed legal challenges to FDA's assertion of jurisdiction. The companies denied that nicotine was addictive and that they had manipulated nicotine levels in the design and manufacture of cigarettes and smokeless tobacco products. In 2000 the US Supreme Court ruled that the FDA did not have authority to regulate tobacco products. In essence, the Court ruled that Congress never intended the FDA to have such authority. This was a ruling on the law, not on the science of nicotine psychopharmacology. In fact, the Court recognized the strength of the science and magnitude of the health problem. Nevertheless, it concluded that only Congress could resolve the issue of how, if at all, tobacco products should be regulated by FDA (Kessler 2001). Since the demise of the FDA tobacco program, several legislative efforts to grant FDA authority to regulate tobacco were mounted. At the time of this writing, none had succeeded though legislation pending in the Congress in 2008 seems to have a better chance of passing than any prior efforts (Pertschuk 2001; Kessler 2001; Roemer 2004; Mullins 2004).

7 Tobacco Regulation in the Twenty-First Century

The FDA investigation established a strong science base for regulating tobacco products. FDA's efforts, along with litigation against the tobacco industry, which uncovered millions of pages of formerly secret tobacco industry documents, together revealed the breadth and depth of the industry's knowledge about the addictiveness of nicotine (Kessler 2001; Daynard 2004; Hurt and Robertson 1998; Slade

et al. 1995). This knowledge, in turn, provided the basis for numerous countries and regions (e.g., Australia, Canada, European Union, India, Japan, United Kingdom) to accelerate their efforts to regulate tobacco products (Gray 2004; Roemer 2004; Borland and Davey 2004; Jha et al. 2004; Royal College of Physicians 2000; De Beyer and Brigden 2003; World Health Organization 2004, 2007).

In the 1990s, the WHO declared that reducing tobacco use was one of its major priorities. Several conferences were held yielding consensus reports. (World Health Organization 1999, 2001) In 2000, work began on the development of an international treaty ("Framework Convention"), which would be the first United Nations treaty ever developed and negotiated by the WHO (http://www.who.int/tobacco/areas/framework/signing_ceremony/countrylist/en/). WHO also established a Scientific Advisory Committee on Tobacco Product Regulation (SACTob), which developed scientific recommendations concerning tobacco product pharmacology, toxicology, measurement issues, and regulatory implications (WHO, SACTob Recommendations, 2002–2003). The advisory committee was "upgraded" and reestablished as the WHO Study Group on Tobacco Product Regulation (TobReg) in 2004. It issued a recommendation for regulating tobacco product ingredients, emissions, and their measurement (World Health Organization Study Group on Tobacco Product Regulation 2004). In turn, WHO collaborated with the US Centers for Disease Control and Prevention, the National Cancer Institute, and the National Institute on Drug Abuse, to establish the International Network for Tobacco Testing and Research for Regulation (INTTARR) in 2004 (Goldberg 2004).

In all of these regulatory efforts, psychopharmacology research played a key role. It established that the nicotine in tobacco was addictive and a major determinant of tobacco use patterns, including withdrawal symptoms. The research helped to understand the complex interactions between the product, environmental factors and individual factors, e.g., how tobacco-associated stimuli such as advertising might elicit craving in a person trying to quit smoking.

8 Research Challenges Critical to Policy Development and Regulation

Former United States Surgeon General, Dr. C. Everett Koop, has expounded on the scope of the public health problem, the importance of the science foundation and the enormous challenges facing tobacco/nicotine researchers and policy makers in the twenty-first century (Koop 2003, 2004; Henningfield and Zeller 2002). He framed the progression of tobacco-related disease by observing that the dawn of the twentieth century was a time in which serious tobacco disease was relatively rare and not recognized as a major public health problem. By contrast, at the start of the twenty-first century it accounted for 20% of all deaths in the United States and was one of the leading causes of death worldwide. Dr. Koop cited WHO estimates of one billion premature tobacco-caused deaths among existing smokers in the twenty-first

century (World Health Organization 2001) and the recognition of the World Bank that tobacco-caused disease would significantly challenge the economic health of developing nations (World Health Organization 1999). Dr. Koop called for accelerated research and health policy change. His goal is to make the twenty-first century the time to reverse this global epidemic. In his vision, research is the pivotal "supportive companion of our public health efforts" to reduce tobacco-caused disease.

For example, our understanding of the contribution of age, nicotine dosing patterns, and environmental stimuli to the dependence process is vital for more effective prevention and treatment and is being addressed in laboratory psychopharmacology research (Palmatier et al. 2006; Shram et al. 2008).

Among the many research challenges and priorities discussed by Koop, and also articulated by the WHO, are several within the domain of psychopharmacology researchers (World Health Organization Study Group on Tobacco Product Regulation 2004; World Health Organization 2001). Progress on these topics could guide more rational policy and regulation, and thereby contribute to improved public health. Key psychopharmacology research challenges include those summarized below.

8.1 The Importance of Dose

Of no surprise to psychopharmacologists, drug dose can determine the nature as well as magnitude of drug effects (Griffiths et al. 1980; USDHHS 1988). This is evident with nicotine across a broad range of physiological and behavioral responses, as documented with both physiological and behavioral measures (Henningfield and Woodson 1989).

Tobacco companies have long investigated the importance of nicotine dosing in their products, driven by their understanding that the development and maintenance of addiction could be facilitated by efforts to ensure that doses would be adequate for the population targeted by the product, that doses too low would not sustain behavior, and that doses too high might be associated with unpleasant effects (Food and Drug Administration 1995, 1996; Henningfield 2004; Kessler 2001; World Health Organization 2001). In the case of smokeless tobacco, nicotine dosing is controlled by factors including variation of nicotine content, pH and buffering capacity of the product, and size of the tobacco cuttings (Fant et al. 1999; Food and Drug Administration 1995, 1996; Henningfield et al. 1995; Djordjevic et al. 1995). In cigarettes, the nicotine dose to which a human smoker is exposed is a complex function of nicotine content of the product, filtration, air ventilation, moisture content, and many other design factors and ingredients (Browne 1990; Food and Drug Administration 1995, 1996; Henningfield 2004; National Cancer Institute 1996, 2001; World Health Organization 2001, 2004).

In the light of the foregoing, it should come as no surprise that questions abound regarding the best way to measure the nicotine dose of cigarettes, e.g., cigarette content versus machine delivery versus bioavailability (Henningfield et al. 1994), and

the measurement and communication of cigarette dosing characteristics remain important scientific challenges (Food and Drug Administration 1995, 1996; National Cancer Institute 1996, 2001; World Health Organization 2004, 2007). What is clear is that the machine-determined cigarette delivery ratings of nicotine (as well as "tar" and carbon monoxide) do not provide accurate or meaningful information about the doses to which cigarette smokers are actually exposed (Food and Drug Administration 1995, 1996; National Cancer Institute 1996, 2001; World Health Organization 2004, 2007). An implication of this is that cigarette brand descriptors that flow from presumptions about dosing characteristics (e.g., light, low, mild, reduced tar) are virtually meaningless with respect to human exposure and disease risk (National Cancer Institute 2001; World Health Organization 2001, 2004, 2007). Presently, WHO and other organizations oppose cigarettes descriptors such as "light," "low," or "reduced" in delivery of "tar" and nicotine because it appears that consumers can achieve highly toxic and equally addictive levels of the substances from the vast majority (if not all) of the cigarettes so labeled. Furthermore, there is no evidence of a health benefit by switching from regular delivery cigarettes to cigarettes with descriptors implying lower delivery.

In addition to questions about how best to measure and communicate tobacco product dosing characteristics are the questions about the many potential ways that tobacco companies control nicotine dosing characteristics to enable consumers to obtain their preferred doses. The tools for dose manipulation at their disposal have been extensively researched by the companies, but which combination of tools they actually employ in a given cigarette brand are not disclosed by the companies (Food and Drug Administration 1995, 1996), e.g., nicotine content versus ingredients to alter delivery versus designs to enable flexible dosing.

How to measure, label, and regulate should ideally start from data concerning the nature and best way to characterize the dose-response relationships. For example, Henningfield et al. (1994) proposed that nicotine level labeling be based on a combination of content and yield data, but verified by bioavailability studies, following the precedents from pharmaceutical labeling in which the labeled drug dose is often based on one or more of these variables. But, in order to act on this proposal additional data about the control and measurement of dosing would need to be generated.

Even cigarette with labels suggesting that they contain no nicotine are not what they seem. For example, cigarettes have been marketed as "denicotinized" and "nicotine free" even though they contain and deliver nicotine (Butschky et al. 1995; National Cancer Institute 2001). Additionally, a proposal to gradually reduce nicotine addiction by gradually restricting the amount of nicotine over time, was considered by the FDA, and subsequently endorsed by the American Medical Association (Benowitz and Henningfield 1994; Henningfield et al. 1998). The FDA had earlier determined that implementation of such an approach was premature, in part on the basis of insufficient evidence concerning the nonaddictive dose threshold; there were concerns that a cigarette that was unable to sustain addiction in adult smokers might be an ideal gateway to addiction for young people by providing doses sufficient to initiate the process of addiction in this population (Food and Drug

Administration 1995, 1996; Henningfield et al. 1998). These and other questions of relevance to public health flow from the poor current understanding of the control and best means to assess nicotine dosing characteristics.

8.2 Abuse Liability as a Function of the Formulation: Implications for Consideration of Controlled Substance Scheduling

The abuse liability of nicotine varies widely as a function of its formulation and speed of delivery. For example, in a cross-study comparison of abuse liability data, Henningfield and Keenan concluded that abuse liability was related to the speed of nicotine delivery and the nature of the nicotine formulation (Henningfield and Keenan 1993). This finding is consistent with data concerning other substances of abuse (Stitzer and De Wit 1998). Every medicinal nicotine product approved by the FDA has been considered for potential labeling and restrictions based on its presumed abuse liability.

Abuse liability concerns date back to the original approval of nicotine gum in 1983. FDA's Drug Abuse Advisory Committee chair, Robert Balster, summarized the committee's recommendation prophetically as follows (Balster 1983): "We are on the horns of a dilemma. The dilemma is posed by the Controlled Substance Act and an apparent need to control nicotine (as a controlled substance, i.e., addictive drug)." Given all of the data, including what appeared to be remarkably low toxicity and addictive potential of the gum, the committee recommended leaving the gum "uncontrolled," but subject to FDA's oversight concerning prescription drugs. The agency ultimately concurred and determined that prescription control would be adequate and did not recommend scheduling. Several reviews of the Controlled Substance Act provisions, its relations to international drug control, and the importance of abuse liability research are available (Balster and Bigelow 2003; Schuster and Henningfield 2003; Spillane and McAllister 2003).

Following several years of marketing and additional research, the agency determined that the abuse liability was sufficiently low as to not pose a barrier to over-the-counter marketing, which was allowed in 1996. The issue arose afresh, however, when the marketer of nicotine gum proposed to market a mint-flavored version of the gum. The FDA raised the possibility that the mint formulation might be of increased abuse liability and required an abuse liability study to help resolve the issue (Houtsmuller et al. 2002). The study showed that although the flavor was preferred, evidence of actual abuse liability remained very low (Houtsmuller et al. 2002). Similarly, when nicotine was formulated in a lozenge to make it easier to use orally than gum (and enable absorption of a somewhat higher fraction of its dose), FDA required an abuse liability study. Again the study showed very low level of abuse liability (Houtsmuller et al. 2003). Nicotine patches did not appear to be seriously considered for Controlled Substance Scheduling but laboratory data were considered in determining that they did not require a "dependence" warning (Pickworth et al. 1994).

The nicotine medication raising the highest level of concern was nicotine nasal spray, which did produce a small elevation in measures of abuse liability relative to nicotine gum, albeit much smaller than for cigarettes (Schuh et al. 1997). These data and clinical trial data indicating prolonged use, and descriptions of effects by some patients as a "rush," led FDA to consider recommending scheduling under the provisions of the Controlled Substances Act (Food and Drug Administration 1995). Evaluation according to the criteria of the Controlled Substance Act (Balster and Bigelow 2003; Food and Drug Administration 1995) suggested that nicotine nasal spray warranted scheduling as a Controlled Substance in Schedule III (i.e., like pentobarbital which is regulated less restrictively than Schedule II morphine and more restrictively than Schedule IV diazepam). Ultimately, NIDA, DEA, and FDA concurred that prescription monitoring with labeling warning of abuse potential between that of nicotine gum and cigarettes would be an adequate level of control and the FDA did not recommend scheduling.

The reason that this is a major issue, however, is that there is a strong clinical and public health rationale to develop nicotine delivery systems that more closely approximate cigarettes with respect to nicotine delivery, acceptability, and pleasure (Warner et al. 1997, 1998; Slade and Henningfield 1998; Henningfield and Slade 1998). Such a product would have a higher abuse liability than even the nasal spray. The serious possibility of CSA-mandated scheduling has substantially reduced commercial interest in the development of such a medication. Yet there has been very little study of the actual abuse liability of substantially more aggressive nicotine delivery systems, including lung delivery, except for tobacco products. It may be that no pure nicotine delivery system would achieve the level of abuse liability of the cigarette. At present, however, regulatory guidance and product development would be operating more on the basis of theory than empirical science. In such a vacuum, regulators are reluctant to draw conclusions and pharmaceutical developers are hesitant to invest the time and money to develop a treatment product whose use might be so severely constrained.

8.3 Tobacco Products are Chemical Cocktails: Drug Interaction Research is Critical

In the 1970s, Murray Jarvik and others, postulated that the powerful reinforcing effects of tobacco for humans were most likely explained by substances in addition to nicotine in the tobacco and smoke (Jarvik 1977; Russell 1979). Addiction to tobacco has been designated by the nomenclature as "nicotine dependence" and "nicotine withdrawal" by the American Psychiatric Association since 1987 because of the presence of nicotine (American Psychiatric Association 1994, 1987; Stratton et al. 2001). In fact, tobacco products are complex chemical cocktails, with the modern commercial cigarette representing perhaps the most complex of all. Therefore, there is also strong merit in the approach of the WHO in its International Classification of Diseases to use the terms "tobacco dependence" and "tobacco withdrawal."

(World Health Organization 1992) This highlights the fact that the pharmacological effects of tobacco are due to a complex mixture of substances. There are many challenges to regulating such a complex mixture, as have been discussed by the WHO, an Institute of Medicine Report, and the WHO TobReg Study Group. (World Health Organization 2001; Stratton et al. 2001; World Health Organization Study Group on Tobacco Product Regulation 2004).

There is a strong rationale for the possibility that the addictiveness and toxicity of tobacco products could be substantially reduced by restricting certain ingredients and design features (Henningfield and Zeller 2002; Henningfield et al. 2004; Myers 2002; Vagg and Chapman 2005). For example, preliminary animal research suggests that acetaldehyde combined with nicotine makes cigarettes more addictive than nicotine alone (DeNoble and Mele 2005). More recent research supports this conclusion (Talhout et al. 2007). Furthermore, it is also increasingly clear that other substances added to cigarettes make the physical act of smoke inhalation more pleasant and can increase the risk of developing addiction and other diseases (World Health Organization 2007). If this research is firmed up, it is plausible to envision that cigarettes could perhaps be made less addictive by restricting or eliminating the use of additives that have synergistic effects with nicotine (Henningfield et al. 2004; Henningfield and Zeller 2002).

In principle, this is an extension of current drug regulation approaches, which make approval and/or the level of control dependent upon design features of the product. In practice, tobacco products, especially cigarettes, are much more complex in their ingredients and designs than many drug products. There are many ingredient combinations and design features of tobacco products that require study to guide such a regulatory strategy. Some of these will involve determining the effects of product manipulations on reinforcing efficacy, discriminative effects, and subjective response.

9 Conclusions

It is difficult to overstate the value of psychopharmacology research and of venues for intellectual exchange such as scientific meetings and journals in the development of national and global policy to control tobacco. As a sign of growth of tobacco dependence-related research, the 1990s gave birth to the Society for Research on Nicotine and Tobacco and its journal, *Nicotine and Tobacco Research*, as venues extending the range of earlier pivotal scientific meetings such as the American Psychological Association and journals such as *Psychopharmacology* (Drobes and Klein 2004; Pomerleau and Hughes 2005). Psychopharmacology research has also laid the foundation for tobacco product control and regulation that is poised to reverse the course of the global epidemic caused by tobacco use. The viability of this goal flows from a foundation of decades of research and progress but, as evidenced by this article and summarized by Dr. Koop, vital questions remain to be resolved (Koop 2003, 2004).

In fact, as described by Koop (2003, 2004), the accomplishments have been many. The progress was rapid, enabled in part by earlier decades of method development, which was readily applied to the study of tobacco and nicotine. The remaining challenges are perhaps even more daunting, however, as they move in part into territory that is less well-defined, such as investigating the psychopharmacology of the complex mixtures comprising tobacco products and their smoke. Fortunately, there is now a broader base of funding support worldwide, an emerging research infrastructure, and the lure of challenging research. Above all else, there is the potential for enormous public health and social benefit.

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