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Lucyna Antkiewicz-Michaluk
Hans Rommelspacher *Editors*

Isoquinolines and Beta-Carbolines as Neurotoxins and Neuroprotectants

New Vistas In Parkinson's Disease Therapy

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Preface

The specific topic "Isoquinolines and Beta-Carbolines as Neurotoxins and Neuroprotectants: New Perspectives in Parkinson's Disease Therapy," was chosen in light of accumulating neurobiological evidence indicating that, in addition to exogenous neurotoxins (e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]), endogenous compounds may play an important role in the most common neurodegenerative disorders (e.g., Parkinson's disease). Two groups of amine-related compounds, which appeared chemically like MPTP, were detected in human brain and cerebrospinal fluid (CSF): β-carbolines (BCs) and tetrahydroisoquinolines (TIQs). These are heterocyclic compounds formed endogenously from phenylalanine/tyrosine (TIQs) and tryptophan, tryptamine, and 5-hydroxytryptamine (BCs), respectively, and exert a wide spectrum of psychopharmacological and behavioral effects. The TIQs and BCs may bind to their own high-affinity sites on neuronal membranes associated with or located close to the receptors of neurotransmitters. Research on TIQs and BCs is stimulated also by their possible role in pathological conditions, especially parkinsonism and alcoholism. Recently, clinical interest has been spurred by their role as neuroprotective, and even neurorestorative, anticonvulsant, and antiaddictive, substances.

In this book we are going to summarize, for the first time, the results from behavioral, neurochemical, and molecular experiments, which demonstrate a wide spectrum of TIQs and BCs effects – from their rather mild neurotoxic actions to the important neuroprotective and antiaddictive properties.

Additionally, the recent results of experimental studies in vivo have allowed a much better understanding and simultaneous comparison of the neurochemical and molecular mechanisms underlying the neuroprotective and neurotoxic actions of endogenous TIQs and BCs and have pointed to the possibility of their therapeutic applications in neurodegenerative diseases such as Parkinson's disease.

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Part I
Isoquinolines as Neurotoxins and Neuroprotectants

Chapter 1
Two Faces of 1,2,3,4-Tetrahydroisoquinoline Mode of Action in the Mammalian Brain: Is It an Endogenous Neurotoxin or a Neuromodulator?

Elżbieta Lorenc-Koci

Contents

Abstract 1,2,3,4-Tetrahydroisoquinoline (TIQ) is the simplest representative of the family of non-catechol TIQs being present naturally in plants and in a variety of food products as well as in the brain of humans, primates, and rodents. Concentration of this compound in the mammalian brain is very low (0.5–10 ng/g), therefore, its determination required a more sensitive method than that for the measurement of classical neurotransmitters. The physiological role of TIQ has not been elucidated so far, but due to similarity of its chemical structure to MPTP, it was proposed to be

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an endogenous neurotoxin involved in the pathogenesis of Parkinson's disease (PD). In order to characterize TIQ properties in the brain, this review has summarized important aspects concerning the possible pathways of its synthesis, distribution, and metabolism in the mammalian organisms. A special attention has been focused on behavioral and neurochemical effects produced by TIQ administered, acutely and chronically, at pharmacological doses to rodents and monkeys. Since TIQ implication in PD is not clear, evidence indicating that it can induce some parkinsonian-like changes in animals and those suggesting that TIQ can act as a modulator of dopaminergic neurotransmission are thoroughly discussed. Finally, as more recent studies have indicated that TIQ can act as a neuroprotective agent, also these experimental data were carefully analyzed. We hope that this review can shed a new light on TIQ mode of action in the mammalian brain.

Keywords 1,2,3,4-Tetrahydroisoquinolines • Rat brain • Dopamine metabolism • Nitric oxide • Glutathione • Neurotoxin • Neuroprotection

1.1 Introduction

Tetrahydroisoquinolines (TIQs) are a big family of compounds widespread in plant and animal kingdoms (McNaught et al. 1998; Rommelspacher and Susilo 1985; Zarranz de Ysern and Ordonez 1981). In general, TIQs can be formed as condensation products of biogenic amines (i.e., phenylethylamines and catecholamines) with aldehydes or α-keto acids by the so-called Pictet–Spengler reaction (Rommelspacher and Susilo 1985; Zarranz de Ysern and Ordonez 1981; Nagatsu 1997; McNaught et al. 1998), although some of them are also synthesized enzymatically (Yamakawa and Ohta 1997; Yamakawa et al. 1999; Naoi et al. 2004). Depending on the chemical structure of biogenic amines participating in these reactions, TIQs family can be divided into compounds with catechol and non-catechol structures.

For the first time, TIQs attracted a considerable attention of neurochemists and pharmacologists when Davis and Walsh (1970a) demonstrated that the alcohol metabolite acetaldehyde promoted in vitro conversion of [^{14}C]dopamine into [^{14}C] tetrahydropapaveroline (THP). Simultaneously, THP was identified in the urine of parkinsonian patients on L-DOPA medication (Sourkes 1971; Sandler et al. 1973; Matsubara et al. 1992) and in the urine and brain of rats treated with L-DOPA (Turner et al. 1974). Almost at the same time, salsolinol (6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline), an adduct of dopamine and acetaldehyde, was determined in urine of non-pathologic human volunteers, occurring at high concentrations in the urine of intoxicated alcoholics (Collins et al. 1979) and in brains of rats treated with ethanol (Collins and Bigdeli 1975). Moreover, 3′,4′-deoxynorlaudanosolinecarboxylic acid (DNLCA), a TIQ derived from dopamine and phenylpyruvic acid, was detected in the urine of phenylketonuric children and in the brain of rats with experimentally induced hyperphenylalaninemia (Lasala and Coscia 1979).

These findings led researchers to suppose that TIQs may play some role in pathological conditions especially in alcoholism, parkinsonism, and phenylketonuria (Davis and Walsh 1970a, b; Nagatsu and Hirata 1987; Lasala and Coscia 1979). However, despite ongoing efforts, the contribution of TIQ to the pathogenesis of these diseases has not been evidenced as yet. Also, their physiological role in the nervous system has not been elucidated so far.

This chapter reviews some important aspects concerning the chemistry, distribution, and pharmacology of 1,2,3,4-TIQ, the simplest representative of the unsubstituted non-catechol TIQs in the mammalian brain, on the background of other compounds from this group. Although TIQ has been proposed to be one of the etiological factors of Parkinson's disease (PD), its implication in the pathogenesis is not clear. Hence, in this chapter both evidence indicating that TIQ can induce some parkinsonian-like changes in animals and those suggesting that it can act as a neuromodulator are thoroughly discussed.

1.2 Chemical Structure and Origin of 1,2,3,4-Tetrahydroisoquinoline in the Brain

1,2,3,4-TIQ is the simplest representative of the group of non-catechol TIQs which occur naturally in plants and in a variety of food products (Makino et al. 1988; Niwa et al. 1989b) as well as in the brain of humans, primates, and rodents (Kohno et al. 1986; Makino et al. 1988; Niwa et al. 1987, 1989a; Ohta et al. 1987; Yamakawa et al. 1999). Apart from TIQ, this group also encompasses other TIQ derivatives, such as 1-methyl-1,2,3,4-tetrahydroisoquinoline (1-MeTIQ), 2-methyl-1,2,3,4-tetrahydroisoquinoline (2-MeTIQ), 1-methyl-3,4-dihydroisoquinoline (1-MeDIQ), 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1-BnTIQ), 1-(3′,4′-dihydroxy-benzyl)-1,2,3,4-tetrahydroisoquinoline [1-(3′,4′-DHBn)TIQ], 1-phenyl-1,2,3,4-tetrahydroisoquinoline (1-PhTIQ), and 1-phenyl-2-methyl-1,2,3,4-tetrahydroisoquinoline (1Ph-2MeTIQ) (Fig. 1.1). TIQs were detected in plants much earlier, before they were found in humans and animals (Rommelspacher and Susilo 1985; Zarranz de Ysern and Ordonez 1981). Finally during the late 1980s, TIQ was identified as an endogenous compound in the brain of parkinsonian patients and normal human subjects, using the most suitable method of gas chromatography–mass spectrometry (GC/MS). (Niwa et al. 1987, 1989a). Its concentration determined for the first time in the frontal cortex of one parkinsonian patient was approximately 10 ng/g vs. less than 1 ng/g in the control brain (Niwa et al. 1987) (Table 1.1). However, a high TIQ content in parkinsonian patients was not confirmed by Ohta et al. (1987). What is more, a tendency for the TIQ concentration to be lower in PD than in the controls (0.54 ng/g vs. 0.86 ng/g, respectively) was described by other researchers (Yoshida et al. 1993). Applying the same analytical method, TIQ was determined in the brain of healthy, nontreated rats in which its level oscillated from 5 to 7 ng/g tissue (Kohno et al. 1986; Makino et al. 1988). However, when a highly sensitive high-performance

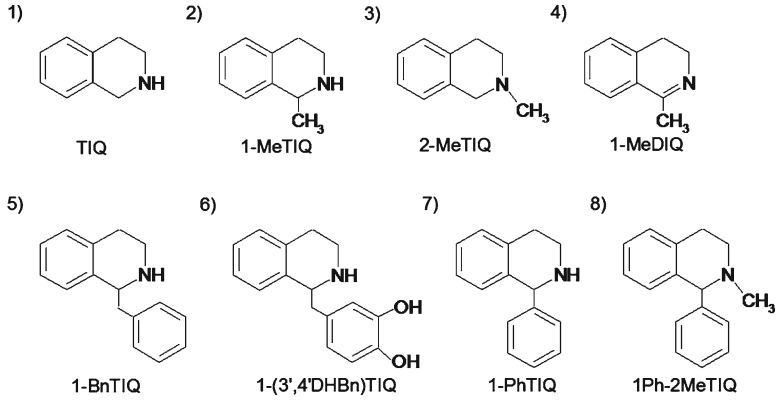

Fig. 1.1 Chemical structure of the non-catechol tetrahydroisoquinolines identified in the mammalian brains: (1) TIQ, 1,2,3,4-tetrahydroisoquinoline; (2) 1-MeTIQ, 1-methyl-1,2,3,4-tetrahydroisoquinoline; (3) 2-MeTIQ, 2-methyl-1,2,3,4-tetrahydroisoquinoline; (4) 1-MeDIQ, 1-methyl-3,4-dihydroisoquinoline; (5) 1-BnTIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline; (6) 1-(3′,4′-DHBn) TIQ, 1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline; (7) 1-PhTIQ, 1-phenyl-1,2,3,4-tetrahydroisoquinoline; and (8) 1Ph-2MeTIQ, 1-phenyl-2-methyl-1,2,3,4-tetrahydroisoquinoline

liquid chromatography with fluorescent detection (detection limits 8–9 fmol per injection) was used, TIQ content in the brain of normal rats was assessed to be much lower reaching an average value of 0.10 ng/g (0.7 pmol/g) tissue (Inoue et al. 2008). Regarding the presence of TIQ in the nigrostriatal dopaminergic system, it was indentified in the substantia nigra (SN) and striatum of rats and monkeys (Yoshida et al. 1990; Ayala et al. 1994; Yamakawa et al. 1999). In either species, in young animals its content was much higher in the SN than in the striatum (Table 1.1). In contrast, in old rats a declining tendency in TIQ concentration was observed in the SN while an increasing trend was characteristic of the striatum (Ayala et al. 1994).

Concentrations of two other non-catechol TIQs, 1-MeTIQ and 1-BnTIQ, identified by means of chromatographic methods in the brains of humans, monkeys, and rodents as well as in the cerebrospinal fluid (CSF) of parkinsonian patients and healthy controls are compiled in Table 1.1. 1-MeTIQ is considered to be a possible neuroprotective compound (Tasaki et al. 1991; Antkiewicz-Michaluk et al. 2004; Kotake et al. 2005; Okuda et al. 2006) while 1-BnTIQ is suspected to be neurotoxic (Kotake et al. 1995, 1998). Interestingly, 1-MeTIQ amount was reduced in parkinsonian patients and tended to decrease with aging (Ohta et al. 1987). In old rats, a 50% reduction in 1-MeTIQ content was found in the SN while only a small nonsignificant increase was observed in the striatum (Ayala et al. 1994). Moreover, 1-MeTIQ exists in the form of two stereoisomers because of the asymmetric center at C-1. The existence of *R*- and *S*-enantiomers has been confirmed in mouse brain applying GC/MS with negative ion chemical ionization (Makino et al. 1990). The proportion of *R*- and *S*-enantiomers in the mouse brain was 0.60 suggesting that 1-MeTIQ could be synthesized, at least partially, in an enzymatic pathway

Table 1.1 Concentrations of TIQ and its derivatives in the brains and cerebrospinal fluid of humans, monkeys and rodents.

TIQ derivatives	Origin of tissue	Type of tissue	Concentration	Method of detection	References
	Human				
TIQ	Control patient	Frontal cortex	~ 1ng/g	GC/MD	Niwa et al. 1987, Niwa et al. 1989a, Ohta et al. 1987
	Parkinsonian patient	Frontal cortex	0.54-10 ng/g		
	Monkey	Striatum	~ 20 pmol/g	GC/MS	Yamakawa et al. 1999
		SN	~ 150 pmol/g		
	Rat	Brain	5-7 ng/g	GC/MID	Kohno et al. 1986,
		Brain	0.7 pmol/g (0.10 ng/g)	HPLC/FD	Makino et al. 1988 Inoue et al. 2008
	Young rats	Striatum	~ 0.6 ng/mg	GC/MS	Ayala et al. 1994
		SN	~ 1.7 ng/mg		
	Old rats	Striatum	~ 1.0 ng/mg		
		SN	~1.3 ng/mg		
	Mouse	Brain	1.1 ng/g	GC/MS	Makino et al. 1988, Tasaki et al. 1991
1-MeTIQ	Human	Brain	–	–	Ohta et al (1987
	Monkey	Striatum	300 pmol/g	GC/MS	Yamakawa et al. 1999
		SN	470 pmol/g		
	Rat	Brain	1-3 ng/g	GC/MID	Kohno et al. 1986
				GC/MS	Makino et al. 1988
			3.4 pmol/g (0.1 ng/g)	HPLC/FD	Inoue et al. 2008
	Young rats	Striatum	~ 0.4ng/mg	GC/MS	Ayala et al. 1994
		SN	~ 1.3 ng/mg		
	Old rats	Striatum	~ 0.5 ng/mg		
		SN	~ 0.6 ng/mg		
	Mouse	Brain	8.9-10 ng/g	GC/MS	Kotake et al. 1998, Tasaki et al. 1991, Makino et al. 1990
	Human				
1-BnTIQ	Control patient	CSF	0.4 ng/ml	GC/MID	Kotake et al. 1995
	Parkinsonian patient	CSF	1.17 ng/ml	GC/MID	
	Monkey	Striatum	~ 25 pmol/mg	GC/MS	Yamakawa et al. 1999
		SN	~ 120 pmol/mg		
	Rat	Brain	1.3 pmol/g 0.3 ng/g	HPLC/FD	Inoue et al. 2008
	Mouse	Brain	5.7 - 7.7 ng/g	GC/MS	Kotake et al. 1995, 1998

GC/MID – gas chromatography with multiple ion detection
GC/MS – gas chromatography with mass spectrometry
FD – fluorometric detection

(Makino et al. 1990). If the formation was purely nonenzymatic, then a mixture of racemic isomers would be formed. Apart from the above-mentioned TIQ derivatives, brains of parkinsonian patients were also shown to contain 1-PhTIQ and

1Ph-2MeTIQ using gas chromatography–tandem mass spectrometry (Kajita et al. 1995). In turn, in the mouse brain 1-(3′,4′-DHBn)TIQ was identified as an endogenous compound by means of the GC/MS method (Kawai et al. 1998, 2000). Since concentrations of the latter compounds were not determined, they are not presented in Table 1.1. However, it is believed that they exert toxic effects on dopaminergic neurons (Kajita et al. 1995; Kawai et al. 1998, 2000).

1.3 Synthesis of Non-catechol 1,2,3,4-Tetrahydroisoquinolines

It is widely accepted that TIQs are formed by a well-known Pictet–Spengler condensation of 2-phenylethylamine (PEA) or catecholamines with aldehydes or α-keto acids (Deitrich and Erwin 1980; Rommelspacher and Susilo 1985; Zarranz de Ysern and Ordonez 1981; Nagatsu 1997; McNaught et al. 1998; Kotake et al. 1998). The reaction is thought to proceed through a Schiff's base formation and cyclization to TIQs (Fig. 1.2). In general, the synthesis of catechol-bearing TIQs under physiological conditions was demonstrated in plants and animals (Zarranz de Ysern and Ordonez 1981; Nagatsu 1997; McNaught et al. 1998). However, the formation of the non-catechol TIQs, such as TIQ and 1-MeTIQ, under physiological conditions seems to be problematic because it has been reported that PEA which has no electron-donating substituents (e.g., hydroxyl or alkoxyl groups) on the phenyl ring, does not cyclize with aldehydes under physiological conditions (Kohno et al. 1986). On the contrary, dopamine, which has an effective substituent (OH group) at the appropriate positions in the phenyl ring, can easily cyclize with aldehydes under physiological conditions. Therefore, if the non-catechol TIQ derivatives are formed in such conditions, it can be assumed that the condensation reaction of PEA with an aldehyde is catalyzed enzymatically. Figure 1.3 shows the proposed synthetic reactions of the non-catechol TIQs. When PEA is condensed with formaldehyde then the simplest TIQ can be formed (Fig. 1.3, reaction 1). The (*R*)-1MeTIQ can be synthe-

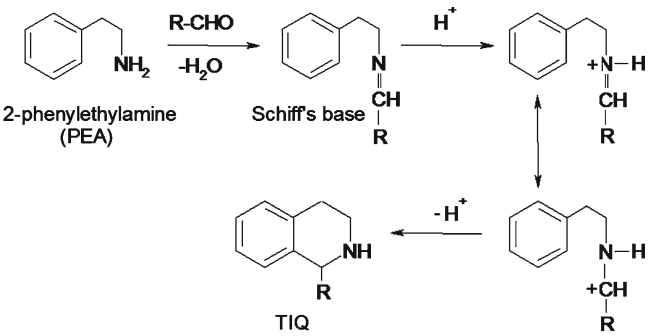

Fig. 1.2 The Pictet–Spengler reaction of β-arylethylamines (2-phenylethylamine, PEA) with carbonyl compounds

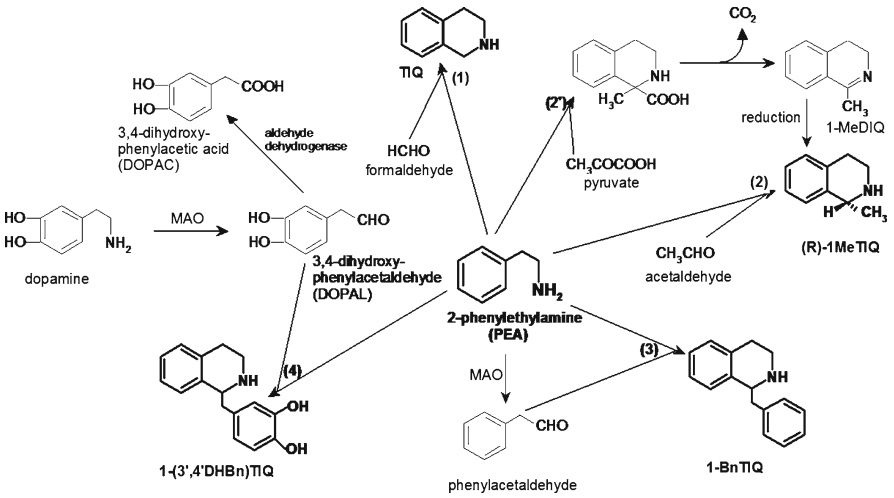

Fig. 1.3 The possible synthetic pathways of the non-catechol tetrahydroisoquinolines: (1) TIQ, 1,2,3,4-tetrahydroisoquinoline; (2) (R)1MeTIQ, (R)-1-methyl-1,2,3,4-tetrahydroisoquinoline; (3) 1BnTIQ, 1-benzyl-1,2,3,4-tetrahydroisoquinoline; (4) 1-(3′,4′-DHBn)TIQ, 1-(3′,4′-dihydroxy benzyl)-1,2,3,4-tetrahydroisoquinoline via the Pictet–Spengler condensation of 2-phenylethylamine (PEA) with aldehydes (formaldehyde, acetaldehyde, phenylacetaldehyde, 3,4-dihydroxyphenylacetaldehyde) or the α-keto acid (pyruvic acid) in the mammalian brain

sized in the condensation reaction of PEA with acetaldehyde or pyruvic acid (Fig. 1.3, reactions 2 and 2′). In the case of reaction 2′, (*R*)-1MeTIQ formation is followed by a decarboxylation of the condensation product, 1-carboxyl-1-methyl-1,2,3,4-tetrahydroisoquinolinic acid and next by a reduction of the second intermediate 1-MeDIQ (Nagatsu 1997). In addition, the condensation reaction of PEA with its own metabolite phenylacetaldehyde or with the dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) may lead to the formation of 1-BnTIQ and 1-(3′,4′-DHBn)TIQ, respectively (Kotake et al. 1998; Kawai et al. 1998). Both phenylacetaldehyde and DOPAL are generated by monoamine oxidase (MAO) during an oxidative deamination of PEA and dopamine, respectively (Fig. 1.3, reactions 3 and 4). It has been demonstrated that the formation of 1-BnTIQ was markedly reduced in the mouse brain treated previously with the MAO-B inhibitor deprenyl (Kotake et al. 1998), possibly due to the deficit of phenylacetaldehyde.

For a long time, an enzymatic biosynthesis of (*R*)-1MeTIQ, from PEA and pyruvate was only a strong suggestion (Makino et al. 1990; Nagatsu 1997), but finally it was confirmed when Yamakawa and Ohta (1997) identified in the rat brain an enzyme involved in this reaction (Fig. 1.4). The 1-MeTIQ synthesizing enzyme was predominantly localized in the mitochondrial–synaptosomal fraction. The activity of this enzyme measured in rat brain homogenate was 750 pmol/h/mg protein (Yamakawa and Ohta 1997). A low activity of 1-MeTIQ synthesizing enzyme was observed in the nuclear fraction, and no activity was detected in the cytosol fraction. In the monkey brain, the 1-MeTIQ synthetic activity was higher in the thalamus, cerebrum, and striatum than in the substantia nigra, medulla oblongata, and cerebellum

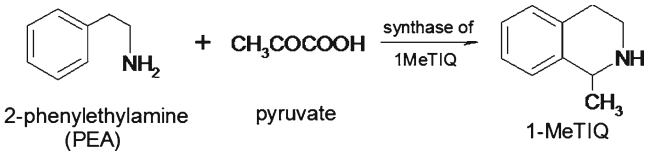

Fig. 1.4 Enzymatic biosynthesis of 1MeTIQ in the mitochondrial–synaptosomal fraction of the rat brain (according to Yamakawa and Ohta 1997)

(Yamakawa et al. 1999). As the content of 1-MeTIQ was the highest in the substantia nigra, striatum, and cerebrum while in the cerebellum, medulla oblongata, and thalamus it was distinctly lower, the authors suggested that 1-MeTIQ which was synthesized elsewhere in the brain was transported to these brain regions.

It has been demonstrated in the mitochondrial–synaptosomal fraction of rat brain that some non-catechol TIQ derivatives, like TIQ, 2-MeTIQ, 1-BnTIQ, and 1-MeDIQ inhibited the biosynthesis of 1-MeTIQ (Yamakawa and Ohta 1997, 1999). Also 1-MeTIQ itself can inhibit an activity of the synthesizing enzyme. The inhibitory activity of *R*-enantiomer of 1-MeTIQ was stronger than that of *S*-enantiomer (Yamakawa and Ohta 1999). In contrast, catechol-TIQ derivatives, like salsolinol and norlaudanosoline weakly inhibited or did not inhibit 1-MeTIQ biosynthesis, respectively (Yamakawa and Ohta 1997, 1999). As the 1-MeTIQ biosynthetic activity was inhibited by non-catechol TIQ derivatives, but was not inhibited by catechol TIQ derivatives, authors of the study postulated that this enzyme was specific for TIQs unsubstituted in the aromatic ring (Yamakawa and Ohta 1997). The activity of the 1-MeTIQ synthesizing enzyme was also inhibited by parkinsonism-inducing substances, like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), haloperidol, and β-carboline (Yamakawa and Ohta 1999). The preservation of the TIQ-generating enzymatic system in the mammalian brain in the course of evolution suggests that it may play an important physiological function.

TIQ identified in the mammalian brain may be also of dietary origin, as it has been detected in different food products, such as cheese (5.2 ng/g), boiled eggs (1.8–2.2 ng/g), banana (2.2 ng/g), broiled beef (1.3 ng/g), milk (3.3 ng/g), and various alcoholic beverages including whisky (0.73 ng/g), wine (0.59 ng/g), and beer (0.36 ng/g) (Makino et al. 1988; Niwa et al. 1989b).

1.4 1,2,3,4-Tetrahydroisoquinoline as a Potential Neurotoxin with a Proparkinsonian Mode of Action

The concept of a TIQ contribution to the pathogenesis of idiopathic Parkinson's disease (PD) sprung from the observation that its chemical structure was similar to MPTP, a selective neurotoxin of dopaminergic neurons which evoked a syndrome

resembling the clinical picture of the disease in humans and animals (Langston et al. 1983; Chiueh et al. 1985). Since MPTP is a synthetic compound, it cannot be considered as an etiological factor for PD, but TIQ which is both an endogenous and an environmental substance seemed to be a good candidate to produce parkinsonism. In principle, the selective toxicity of MPTP is grounded on its oxidative MAO-B-dependent transformation to the quaternary ion MPP^+ (Bradbury et al. 1986; Trevor et al. 1988). Then MPP^+ ion being a substrate for dopamine transporter (DAT) (Javitch et al. 1985) is selectively accumulated in the dopaminergic neurons finally leading to an inhibition of the oxidative phosphorylation at complex I of the mitochondrial respiratory chain and to the reduction of ATP production (Trevor et al. 1987; Singer et al. 1988). It is worth underlining that the presence of N-methyl group is essential for the manifestation of MPTP toxicity, since analogues of MPTP and MPP^+ lacking the N-methyl group are devoid of such an effect (Bradbury et al. 1985). Hence, it was assumed that TIQ, like MPTP, could acquire the neurotoxicity after N-methylation and oxidation. In fact, N-methylation of TIQ to 2-MeTIQ by N-methyltransferase was confirmed in vitro, in experiments with the use of the human brain homogenates (Naoi et al. 1989b) and in vivo in the brain of TIQ-treated monkeys (Niwa et al. 1990). The reaction required *S*-adenosyl-L-methionine (SAM) as a methyl donor and the value of the Michaelis constant, K_m, and the maximal velocity, V_{max}, in terms of SAM were 5.11 μM and 7.31 pmol/min/mg protein, respectively. The value of K_m and V_{max} in terms of TIQ were 20.9 μM and 7.98 pmol/min/mg protein, respectively (Naoi et al. 1989b). Afterwards, it was demonstrated in the human brain synaptosomal mitochondria that 2-MeTIQ could be oxidized by both types, MAO-A and -B into 2-methylisoquinolinium (2-MeIQ^+) ion, an analogue of MPP^+ (Naoi et al. 1989a). MAO type A had a higher activity for 2-MeTIQ than type B. The K_m and V_{max} values of the oxidation by MAO type A and B were 571 μM and 0.29 pmol/min/mg protein, and 463 μM and 0.16 pmol/min/mg protein, respectively (Naoi et al. 1989a). In comparison, the V_{max} value of MAO type A for MPTP was 19.4 pmol/min/mg protein in human brain synaptosomal mitochondria (Naoi et al. 1987). The above-mentioned effects clearly indicated that 2-MeTIQ oxidation was distinctly slower than that of MPTP. Further testing of 2-MeIQ^+ ion mode of action showed in the rat clonal pheochromocytoma PC12h cell line that this compound was transported into cells by a DA-specific uptake system, similarly like MPP^+ (Naoi et al. 1989c). Moreover, 2-MeTIQ and 2-MeIQ^+ ion were reported to selectively inhibit complex I activity of the mitochondrial electron transport system in isolated mitochondria prepared from the mouse brain (Suzuki et al. 1992a). Finally, the selective neurotoxicity of 2-MeIQ^+ toward dopaminergic neurons was demonstrated in the ventral mesencephalic culture (Niijima et al. 1991; Nishi et al. 1994).

All the above-described findings provided grounds for the studies whose aim was to check whether TIQ was able to induce behavioral and neurochemical changes of parkinsonian type in animals.

1.5 Behavioral and Neurochemical Changes of Parkinsonian Type Induced by Non-catechol 1,2,3,4-Tetrahydroisoquinolines in Animals

The major clinical signs of an extrapyramidal syndrome in PD, such as akinesia, and muscle rigidity appear when the level of dopamine (DA) in the caudate-putamen is decreased by 85% and almost 90% of dopaminergic neurons in the substantia nigra (SN) are destroyed (Kish et al. 1988). These symptoms also appear in the MPTP-treated monkeys suffering from more than 80% reduction in the striatal dopamine and from a greater than 80% decrease of dopaminergic cell bodies in the SN (Chiueh et al. 1985).

In the first experiment performed in marmosets treated subcutaneously with TIQ at a high pharmacological dose of 50 mg/kg/day for a period of 16 days, it was demonstrated that the most pronounced motor deficits and muscle rigidity were revealed after the last chronic dose of this compound (Nagatsu and Yoshida 1988). At that time point, in two examined TIQ-treated marmosets, an almost 70% decrease in the level of dopamine (DA) was observed in the substantia nigra (SN) but only in one marmoset some moderate decrease in its content was found in the striatum (Nagatsu and Yoshida 1988). In squirrel monkeys, TIQ administered at a moderate pharmacological dose of 20 mg/kg/day for up to 104 days, produced motor symptoms similar to parkinsonism conspicuous even 7 days after discontinuation of chronic treatment (Yoshida et al. 1990). At that time point, in these monkeys only a 23% decline in the nigral level of DA and no changes in its striatal level were found (Yoshida et al. 1990). In turn, rats chronically injected with TIQ at a dose of 50 mg/kg/day for 19 days exhibited distinct muscle rigidity, observable already 1 h after the first TIQ dose, when there were no changes in the striatal level of DA (Lorenc-Koci et al. 2000). This symptom was still present at 72 h after the last chronic dose of TIQ, but then its expression was less pronounced. At the latter time point, in rats withdrawn from chronic TIQ treatment, hardly a 23% decline in the striatal level of DA was found (Lorenc-Koci et al. 2000). Finally, in C57BL mice injected with the maximal tolerated doses of TIQ (60 up to 150 mg/kg/day) for a period of 26 days, no reduction in the content of DA and its metabolites was reported in the striatum 5 weeks after discontinuation of TIQ treatment (Perry et al. 1988). The only behavioral alteration observed in these C57BL mice was sedation occurring for a short period of time after TIQ injections at a dose of 80 mg/kg or higher (Perry et al. 1988). As results from the above representative studies, TIQ administered chronically at a wide range of doses to monkeys and rodents evoked in these animals moderate small or no changes in DA concentrations in the striatum and SN. In contrast to the effects reported in TIQ-treated animals, even a single dose of MPTP was able to produce in humans a drastic loss of striatal DA and a damage of dopaminergic neurons in the SN (Langston et al. 1983).

Investigations focusing on the potential toxic action of TIQ on the dopaminergic neurons in the SN were performed in C57BL mice treated with TIQ at a dose of 50 mg/kg for 70 days. In these mice, the numbers of tyrosine hydroxylase immunoreactive (TH-ir) neurons in the SN and ventral tegmental area were reduced by 56% when measured 24 h after the last chronic dose of TIQ (Ogawa et al. 1989). Such long-lasting TIQ treatment did not produce, however, the death of DA neurons because cresyl violet (CV) staining revealed that the numbers of CV-stained neurons in the examined structures were almost the same as those of the control mice. So, it was concluded that DA neurons were preserved but they were dysfunctional in terms of their ability to produce TH protein (Ogawa et al. 1989). Our study carried out on rats receiving TIQ at a dose of 100 mg/kg/day chronically for a period of 19 days demonstrated that the number of TH-ir neurons in the SN was also reduced but only by 22% in comparison to control group (Lorenc-Koci et al. 2000). However, because in this study the histological analysis of CV-stained neurons was not performed, it was not possible to resolve whether TH-ir neurons were preserved or not. Moreover, since 2-MeTIQ was recognized as a more toxic derivative than TIQ itself, the effect of this compound on dopaminergic neurons in the SN was examined in C57BL/6J mice. 2-MeTIQ was administered at a wide range of doses (2, 4, 16, 32, and 64 mg/kg) for 120 days. In all groups, 2-MeTIQ evoked a significant decrease in the numbers of TH-ir neurons in the SN, although the effects were more pronounced at higher doses of this compound. However, despite some atrophic changes observed in the nerve cells of the central part of the substantia nigra pars compacta (SNc) and pars lateralis neither neuronal loss accompanied by gliosis nor neuronal inclusions were observed (Fukuda 1994).

All the above-reported effects clearly indicate that the appearance of characteristic symptoms of parkinsonian type in the TIQ-treated animals do not result from the loss of striatal DA and death of dopaminergic cells in the SN, as it happens in PD. Since these symptoms were the most distinctly manifested directly after subcutaneous or intraperitoneal administration of TIQ, when its concentration in the mammalian brain was very high (Yoshida et al. 1990; Lorenc-Koci et al. 2004a), it was assumed that the occurrence of these symptoms was related to a specific TIQ action on dopaminergic neurotransmission. The latter assumption was in line with studies showing that exogenous TIQ easily crossed the blood–brain barrier (Niwa et al. 1988; Yoshida et al. 1990; Kikuchi et al. 1991; Lorenc-Koci et al. 2004a) and interacted with the brain DA receptors (Antkiewicz-Michaluk et al. 2000a). Further, it was demonstrated that TIQ displaced [^{3}H] apomorphine from its binding sites within dopamine D_1 and D_2 receptors with effectiveness similar to DA and in behavior tests inhibited the apomorphine-stimulated locomotor activity (Antkiewicz-Michaluk et al. 2000a). The ability of TIQ to interfere with the agonist binding sites within DA receptors inhibiting their function suggests that this compound can attenuate the dopaminergic neurotransmission at sites other than those to which classical neuroleptics bind. So, it is likely that the neuroleptic-like activity of TIQ was responsible for some motor deficits observed in acutely and chronically TIQ-treated animals.

1.6 Catabolism of 1,2,3,4-Tetrahydroisoquinoline in the Brain and Peripheral Tissue

Although PD occurs sporadically, it is believed that both environmental and genetic factors, acting either alone or in concert, contribute to the onset of the disease. Genetic susceptibility to endogenous or exogenous neurotoxins may be related to the altered activity of some enzymes which regulate their metabolism (Riedl et al. 1998). The lack of a metabolic pathway or a deficit in its function may influence toxicity. Among different enzymes which are involved in the metabolism of xenobiotics, cytochrome P 450 (CYP) isoenzyme CYP2D6 was postulated to be a risk factor for PD (Barbeau et al. 1985; Bon et al. 1999; Checkoway et al. 1998; Riedl et al. 1998).

Isoenzymes of the human CYP2D subfamily are encoded by one active *CYP2D6* gene and two pseudogenes, while six genes, *CYP2D1-5* and *CYP2D18*, have been identified in rats (Kimura et al. 1989; Matsunaga et al. 1990). It is still unclear which of these six known rat CYP2D subfamily members are homologous to human CYP2D6. For a long time it was assumed that CYP2D1 corresponded well with human CYP2D6 (Barham et al. 1994; Miksys et al. 2000; Tyndale et al. 1999), but recently it was demonstrated that debrisoquine a classical substrate for CYP2D6 was also metabolized in rats to 4-hydroxydebrisoquine by hepatic CYP2D2 (Hiroi et al. 2002). In humans, CYP2D6 has high debrisoquine 4-hydroxylation activity while in rats this activity was much more specific for CYP2D2 (Schulz-Utermoehl et al. 1999).

MPTP which evokes parkinsonism in humans is metabolized to N-demethyl product by microsomal CYP2D isoenzymes (Coleman et al. 1996; Gilham et al. 1997). Since MPTP lacking N-methyl group does not exert toxic effects, the N-demethylation reaction of MPTP is considered to be detoxification (Coleman et al. 1996; Weissman et al. 1985). On the other hand, female Dark Agouti rats, a model of human poor metabolizer phenotype (PM) with respect to CYP2D6, are more sensitive to neurotoxic effect of MPTP than females of other strains (Jiménez-Jiménez et al. 1991).

The hypothesis put forward by Ohta et al. (1990) linked a potential toxicity of TIQ with its defective catabolism in the liver by isoenzymes belonging to the CYP2D subfamily. According to this hypothesis, the main metabolic pathway of TIQ elimination from the body is the reaction of 4-hydroxylation catalyzed by hepatic CYP2D. The authors reported that after TIQ administration to Dark Agouti rats, plasma and brain levels of this compound were much higher in females of that strain recognized as poor debrisoquine metabolizers than in males considered as extensive debrisoquine metabolizers. Conversely, urinary excretion of a major oxidative metabolite of TIQ, 4-hydroxytetrahydroisoquinoline (4-OH-TIQ) was high in Dark Agouti males while being significantly reduced in females of that strain. Hence, it was concluded that suppression of TIQ metabolism in the liver of poor debrisoquine metabolizers resulted in the increased level of TIQ in the brain. Assuming TIQ toxicity for dopaminergic neurons, it was postulated that a long-lasting accumulation of this compound in the brains of human poor debrisoquine metabolizers may be one of the mechanisms responsible for the onset of PD (Ohta et al. 1990).

To check the accuracy of this hypothesis, the effects of TIQ treatment on the disposition of this compound in the brain was examined in rats being models of poor CYP2D metabolizers (Lorenc-Koci et al. 2004a). Since the inhibition of CYP2D isoenzymes by a specific inhibitor, quinine (Kobayashi et al. 1989), in male Wistar rats mimics the defect of genes encoding CYP2D isoenzymes; the quinine-pretreated Wistars, used in these studies, were considered to correspond to phenotypic poor CYP2D metabolizers. On the other hand, male Dark Agouti rats in which the expression of CYP2D2 isoenzyme was six to eight times lower than that in male Wistars (Schulz-Utermoehl et al. 1999), were used as genotypic poor CYP2D metabolizers (Lorenc-Koci et al. 2004a). Male Wistar rats with normal function of these isoenzymes were the control for phenotypic and genotypic poor CYP2D metabolizers (Lorenc-Koci et al. 2004a). TIQ was administered i.p. to male Wistar rats at doses of 20, 40, and 100 mg/kg, alone and in combination with quinine (20, 40, 80 mg/kg i.p.), acutely or chronically. Only acute experiments were performed in Dark Agouti rats receiving TIQ at doses of 20 and 40 mg/kg. Concentrations of TIQ and its main metabolite 4-OH-TIQ in plasma and brain samples were determined using HPLC with UV detection, described previously by Suzuki et al. (1992b). Both in Wistar and Dark Agouti rats 2 h after administration of a single dose of TIQ (20, 40, 100 mg/kg), the level of this compound in the brain depending of the used dose was in the range from 86 to 682 nmol/g, while in the plasma it was several-fold lower ranging from 24 to 120 nmol/ml. Concentrations of its metabolite, 4-OH-TIQ, were very low in both compartments of male Wistars treated with TIQ alone (in plasma about 1 nmol/ml; in brain 2.46–3.97 nmol/g) while in those receiving TIQ in combination with quinine or in Dark Agouti males, 4-OH-TIQ was absent or found in a trace amount (Lorenc-Koci et al. 2004a). These data clearly indicated that TIQ was not easily metabolized via 4-hydroxylation and this reaction in the liver had no influence on its concentration in the brain. Hence, it was concluded that factors other than CYP2D mediated catabolism contributed to the disposition of TIQ in the rat brain.

It was originally believed that membrane-bound carriers localized in the brain barriers were solely responsible for the transport of endogenous substances into and out of the brain, and that drug transport across the brain barriers was largely dependent on the physiochemical characteristic of the drug, such as lipophilicity, molecular weight, and ionic state (Spector 1990; Tamai and Tsuji 2000). TIQ is a basic compound with pK_a value of 9.75, moderate lipophilicity with a log$P$ value 1.47 (P is an octanol/water coefficient for a nonionized drug, log$P$ was calculated using a special computer program), and low molecular weight (169.99). These physicochemical properties of TIQ should allow for its passive diffusion through the blood–brain barrier. However, a low level of TIQ (about 50 nmol/g) in the brain of Wistar rats receiving this compound (40 mg/kg) jointly with 80 mg/kg of quinine in comparison to its high level (244.94 nmol/g) in rats receiving TIQ alone, suggested that there was a competition between TIQ and quinine for the same carrier (Lorenc-Koci et al. 2004a). Quinine is a substrate for organic cation transporter (OCT) system (Lee et al. 2001), therefore, it was supposed that also TIQ could be transported by this system.

In order to confirm OCT contribution to the transport of TIQ from the periphery into the brain, an experiment with its specific inhibitors was performed in Wistar rats. Three distinct types of the OCT system have been identified (OCT1, OCT2, OCT3) in the rat brain. Acute administration of progesterone (20 mg/kg) and β-estradiol (0.2 and 1 mg/kg), that are inhibitors of OCT1/OCT2 and OCT3 respectively, to Wistar rats 30 min before TIQ, significantly decreased the concentration of TIQ measured 2 h later in the brain tissue. The effect was more pronounced in rats pretreated with β-estradiol than in those pretreated with progesterone (Lorenc-Koci et al. 2004a). The obtained results are in line with the abundant expression of OCT3 and slightly weaker expression of OCT2 in the rat brain (Amphoux et al. 2006; Shang et al. 2003; Wu et al. 1998). From these experiments, it was concluded that exogenous TIQ was actively transported from the blood into the brain by OCT system, mainly by OCT3 (Lorenc-Koci et al. 2004a).

The cited study also revealed that 4-OH-TIQ was formed not only by hepatic CYP2D isoenzymes but also by their brain isoforms. In Wistar rats with normal function of CYP2D isoenzymes, 2 h after the last chronic dose of TIQ (50 mg/kg, two times per day for 14 days), concentrations of this metabolite in the plasma and brain were 2.54 nmol/ml and 11.51 nmol/g, respectively, while concomitant concentrations of TIQ in these compartments were 84.73 nmol/ml and 556.30 nmol/g, respectively. Much higher concentration of 4-OH-TIQ in the brain than in plasma suggested that TIQ was able to induce brain CYP2D isoenzymes. Therefore, it seems that the reaction of 4-hydroxylation although meaningless for elimination of a large amount of exogenous TIQ, may be important for the elimination of TIQ formed endogenously in the brain under physiological conditions.

Concentrations of TIQ and its metabolite were also determined in the dopaminergic structures of normal Wistar rats treated acutely and chronically with TIQ. At 2 h after administration a single dose of TIQ (50 mg/kg), concentration of this compound was almost equal in the striatum and the SN (about 200 nmol/g). However, 2 h after the last chronic dose (50 mg/kg, two times per day for 14 days) the level of TIQ in the SN (415 nmol/g) was about twofold higher than that in the striatum (222 nmol/g). The concentrations of 4-OH-TIQ in the striatum and the SN were 2.23 and 14.98 nmol/g, respectively. TIQ content in either structure distinctly declined 24 h after cessation of chronic treatment (47.69 nmol/g in the striatum and 37.32 nmol/g in the SN), which meant that this compound was relatively easily eliminated from the brain of Wistar rats. The calculated half-life of TIQ in the brain was $t_{1/2}=3.58$ h while its value in plasma was $t_{1/2}=2.38$ h. In turn, in Wistar rats receiving the same dose of TIQ chronically in combination with quinine (40 mg/kg, two times per day for 14 days) the TIQ level was high in both structures, but its distribution was altered (430 nmol/g in the striatum and 229.90 nmol/g in the SN). 4-OH-TIQ was not detected in the structures under study. Moreover, 24 h after withdrawal from the combined chronic treatment, TIQ concentrations in the striatum and SN (125.80 and 78.05 nmol/g, respectively) were markedly higher than those in Wistars receiving TIQ alone (47.69 and 37.32 nmol/g, respectively). The latter effects indicated that the rate of TIQ elimination from the examined structure was distinctly slower in Wistar rats treated with quinine than in those receiving TIQ

alone. Since quinine is an inhibitor of P-glycoprotein (P-gp) which is involved in the extrusion of xenobiotics from the brain (Lee et al. 2001; Silverman 1999), it was thought that P-gp could have contributed to the elimination of TIQ from the rat brain. Indeed, in Wistar rats receiving TIQ, in combination with verapamil, the most specific inhibitor of P-gp, the TIQ concentration measured in the brain tissue 6 h later, was markedly higher than in rats treated with TIQ alone. This experiment confirmed that P-gp contributed to the elimination of TIQ from the rat brain. Hence, it was concluded that the genetic defect of P-gp, but not *CYP2D2/CYP2D6* as postulated previously (Ohta et al. 1990), could favor, if any, the accumulation of TIQ in the mammalian brain (Lorenc-Koci et al. 2004a).

The above-presented study touches a very important problem in the pathogenesis of PD, namely the genetic background underlying the accumulation of specific neurotoxins with proparkinsonian mode of action in the brain dopaminergic structures. The above-reported study suggests that a defective function of P-gp may be a risk factor for PD. This assumption is in agreement with a more recent study by Furuno et al. (2002) who showed that the frequency of 3435T/T genotype, which is associated with a decreased P-gp expression and function, was higher in parkinsonian patients suffering from both early- and late-onset disease than in control. The decreased function of P-gp was also confirmed directly in the brain of parkinsonian patients using [(11)C]-verapamil positron emission tomography (Kortekaas et al. 2005).

1.7 Effect of 1,2,3,4-Tetrahydroisoquinoline Administration on Dopamine Metabolism

The loss of striatal dopamine (DA) in a consequence of degeneration of the nigrostriatal dopaminergic neurons is the most characteristic neurochemical feature of Parkinson's disease. Therefore, early studies, which attempted to demonstrate that TIQ evoked neurochemical changes of parkinsonian type in animals, focused just on determination of the striatal level of DA at different time points after cessation of chronic TIQ treatment (Nagatsu and Yoshida 1988; Yoshida et al. 1990; Perry et al. 1988). However, a direct effect of this compound on DA catabolism was not analyzed in those studies.

It is well known that catabolism of DA to its final metabolite homovanillic acid (HVA) runs both intra- and extraneuronally. DA present in neuronal cytoplasma is N-oxidized by mitochondrial outer membrane enzyme MAO to form 3,4-dihydroxyphenylacetic acid (DOPAC), which is then extraneuronally O-methylated by catechol-*O*-methyltransferase (COMT) to form HVA. DA released into the synaptic cleft may be then taken up by DAT localized on DA terminals or extraneuronally O-methylated by COMT to form 3-methoxytyramine (3-MT) which is then N-oxidized by glial MAO_B to a final metabolite HVA. The formation of DOPAC is accompanied by production of a potent, non-radical oxidant hydrogen peroxide. Its decomposition in the presence of ion-II may be a significant source of the most deleterious radicals that is hydroxyl radicals (Chiueh et al. 1993). Moreover,

hydrogen peroxide can oxidize glutathione (GSH) and other cellular thiols (thioredoxins, cysteine) which are involved in the maintenance of the redox state of cells (Jones 2008; Kemp et al. 2008). Excessive generation of hydrogen peroxide may disrupt the cellular function of these thiols finally leading to pathological changes. Therefore, the oxidative MAO-dependent pathway of DA catabolism may play an important role in the progressive and selective loss of the dopaminergic neurons in the SN during the development of PD. On the other hand, the enhanced catabolism of DA through COMT-dependent O-methylation leading to 3-MT accumulation may constitute an oxidative defense mechanism (Miller et al. 1996).

Due to a short half-life of TIQ in the rat brain ($t_{1/2}$ = 3.58 h), a detailed analysis of DA metabolism in the striatal and nigral homogenates originating from TIQ-treated rats, was performed 2 h after the first and last chronic dose of this compound (50 mg/kg i.p, two times a day for 14 days) (Lorenc-Koci et al. 2004b). This analysis revealed that TIQ administered at a single dose of 50 mg/kg significantly increased the DA level in the striatum and injected chronically, also in the SN. An increasing tendency in DA content was still observed in the striatum 2 h after the last chronic dose of TIQ. As to DA metabolites, TIQ strongly depressed the level of the intraneuronal DA metabolite DOPAC and enhanced that of the extraneuronal 3-MT in the striatum and the SN after either treatments. The level of the final DA metabolite HVA was enhanced only in the striatum after acute treatment, but it was unchanged after chronic treatment in both structures.

The decreased level of DOPAC indicated that the enzymatic activity of both MAO-A and -B that metabolize DA in the rat brain and the DA reuptake system were inhibited by TIQ administration. In turn, the increased level of 3-MT showed that the COMT-dependent pathway of DA catabolism was activated. Moreover, a rapid accumulation of 3-MT indirectly indicated that TIQ was able to release DA in the striatum and SN. The above conclusions drawn from the analysis of DA catabolism were in line with the previous studies which demonstrated that TIQ was an inhibitor both for MAO-A and -B (Maruyama et al. 1993; Patsenka and Antkiewicz-Michaluk 2004) and a substrate for DA re-uptake system (McNaught et al. 1996). The enhanced release of DA (by 280% of basal level) in the striatum of rats receiving a single dose of TIQ (100 mg/kg) was directly confirmed by means of microdialysis method (Lorenc-Koci et al. 2000). In the latter study, it was evidenced that apart from DA, TIQ also released serotonin (5-HT). Extracellular levels of DA and 5-HT metabolites, DOPAC, HVA, and 5-hydroxyindoleacetic acid (HIAA) in the rat striatum were decreased by 40–60% of the basal values. The ability of TIQ to shift DA catabolism from N-oxidation towards O-methylation suggests that it can modulate DA catabolism in a manner similar to MAO inhibitors which are considered as neuroprotective compounds (Magyar et al. 1998; Stern 1998). Such a mode of TIQ action in the rat brain seems to oppose the view that this compound is an endogenous neurotoxin.

It is commonly known that DA is formed from L-tyrosine by two enzymes tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase. In PD, activity of TH, the initial and rate-limiting enzyme in the biosynthesis of DA, was markedly reduced both in the striatum and in the SN, due to degeneration of nigrostriatal dopaminergic

neurons. TIQ administration affected not only DA catabolism but also its synthesis. In early studies, the measurement of TH activity based on DOPA accumulation was performed in marmosets and monkeys chronically treated with TIQ (Nagatsu and Yoshida 1988; Yoshida et al. 1990). In marmosets, it was demonstrated that TIQ decreased TH activity both in the striatum and SN (Nagatsu and Yoshida 1988). However, in the TIQ-treated monkeys, enzymatic activity of TH was unchanged in the striatum though it was markedly reduced in the SN (Yoshida et al. 1990). Histological analysis of TH-ir and CV-stained neurons performed in the mouse SN (Ogawa et al. 1989) suggested that chronic TIQ treatment might lead to the diminution of TH protein production. Therefore, in our study, the level of TH protein was determined by a Western blot method in the striatum and SN of rats chronically treated with TIQ (Lorenc-Koci et al. 2004b). In that study, it was demonstrated that 2 h after the last chronic dose of TIQ, the TH protein level in the striatum was markedly decreased (by 40 % of the control level) though DA content in that structure indicated an increasing tendency. In the SN, although the level of TH protein was unchanged, a marked increase in DA content was observed (Lorenc-Koci et al. 2004b). The TH protein level does not reflect activity of this enzyme, however, a high concentration of DA in the rat striatum and concomitantly decreased level of TH suggested that activity of the remaining part of the enzyme had to be elevated.

TH is an oxidatively labile enzyme whose level of activity is determined by the redox status of its cysteine sulfhydryl groups. Oxidants of –SH groups like peroxynitrite and catechol-quinones reduce TH activity to an extent that is proportional to cysteine modification (Kuhn et al. 1999a, b). Recently, it has been demonstrated that this enzyme was regulated by S-glutathionylation. This redox-sensitive post-translational modification relies upon the reaction in which glutathione disulfide (GSSG) reacts with protein sulfhydryl groups forming protein–glutathione mixed disulfides (Giustarini et al. 2004). When six of seven cysteinyl groups in TH are S-glutathionylated, the activity of this enzyme is lowered by 70–80% (Borges et al. 2002). S-Glutathionylated proteins which are accumulated under oxidative/nitrosative stress conditions can be readily reduced to free –SH groups by glutaredoxin, an enzyme that requires optimal cellular GSH level for its efficient function (Kenchappa and Ravindranath 2003).

Recently, it has been demonstrated that TIQ administration significantly increased the level of reduced GSH in the whole rat brain as well as in its dopaminergic structures, i.e., the striatum and SN (Lorenc-Koci et al. 2005a). In TIQ-treated rats GSH:GSSG ratios in the striatum and SN were significantly higher than in controls, indicating that oxidation/reduction (redox) state of GSH/GSSG couple was shifted in favor of reduction reactions. In such conditions, TH activity could rise in consequence leading to the increased synthesis of DA. In fact, 2 h after the first TIQ dose, a 21% increase in DA content was observed in the rat striatum (Lorenc-Koci et al. 2004b). Then, in chronically TIQ-treated rats, 2 h after the last dose, the striatal concentration of DA was still slightly higher than in control ones though TH level was markedly decreased (Lorenc-Koci et al. 2004b). It seems likely that in these rats due to a long-term maintaining of a high redox state of GSH/GSSG couple and connected with this high activity of TH, a compensatory decline of TH protein level

could occur in order to prevent an excessive production of DA. On the other hand, 3 days after termination of chronic TIQ treatment when the redox state of GSH/GSSG couple returned to control level and TH protein content was still below physiological level, a small 23% decline of DA content in the rat striatum was still present (Lorenc-Koci et al. 2000; Antkiewicz-Michaluk et al. 2000b).

These results indicate that TIQ raising cellular GSH content can affect the activity of TH and possibly other GSH-related enzymes. Hence, the above-reported effects suggest that TIQ through the influence on the redox state of GSH/GSSG couple can modulate DA synthesis in the nigrostriatal dopaminergic system.

1.8 Influence of 1,2,3,4-Tetrahydroisoquinoline on the Levels of Glutathione and Nitric Oxide in the Brain: Neuroprotective Effects in Cell Culture and in Animal Models

Apart from a dramatic loss of DA in the nigrostriatal dopaminergic system in PD, a marked decrease in the concentration of the reduced GSH, the most abundant antioxidant in the mammalian brain, has been reported in the SN (Perry et al. 1982; Sofic et al. 1992; Sian et al. 1994a; Fitzmaurice et al. 2003). The decrease in GSH content is regarded to be an early biochemical marker of PD because it precedes the appearance of the most characteristic biochemical changes visible in the advanced stage of the disease, such as a decline of DA concentration in the striatum, reduction of mitochondrial complex I activity, and alteration of ion metabolism (Pearce et al. 1997; Riederer et al. 1989). The reason for the decline of GSH level in PD has not been elucidated so far. However, it does not seem to result from the decreased synthesis of this antioxidant as the activity of γ-glutamylcysteine synthetase, the rate limiting enzyme in the GSH biosynthesis was not altered in the brain of parkinsonian patients (Sian et al. 1994b). On the other hand, a marked increase of γ-glutamyl transpeptidase (γ-GT) activity, a membrane-bound enzyme responsible for extracellular degradation of GSH, was demonstrated in the SN of PD patients (Sian et al. 1994b). It is assumed that the increase of γ-GT is a compensatory change in response to the loss of GSH in the SN, since cysteine released during the extracellular GSH degradation after uptake into the cell can be reused for de novo GSH biosynthesis.

As mentioned in Sect. 1.7, TIQ administered both acutely and chronically increased GSH level in the whole rat brain as well as in the dopaminergic structures (SN, striatum, and cortex). Moreover, it markedly inhibited the γ-GT enzymatic activity in the studied structures (Lorenc-Koci et al. 2001, 2005a). These results clearly showed that the effects of TIQ on the GSH level and γ-GT activity were in contradiction to the changes observed in PD. TIQ mode of action contrasted also with MPTP activity which reduced GSH content in the nigrostriatal dopaminergic system in mice (Ferraro et al. 1986; Yong et al. 1986; Oishi et al. 1993). Moreover, it was demonstrated that GSH depletion did not cause per se any damage to the

nigrostriatal pathway (Toffa et al. 1997); however, it increased the susceptibility of DA neurons to the toxicity of MPTP and 6-OHDA (Pileblad et al. 1989; Wüllner et al. 1996). In contrast to MPTP, it seems that TIQ rising GSH level in the dopaminergic structures increases the antioxidant capacities of DA neurons and in this way protects them against toxic insults. In line with the latter assumption, to check potential neuroprotective properties of TIQ, the compound in question was administered to rats unilaterally lesioned with disodium malonate (Lorenc-Koci et al. 2005b). Malonate, a reversible inhibitor of the mitochondrial enzyme succinate dehydrogenase (SDH), is frequently used as a model neurotoxin to induce lesion of the nigrostriatal dopaminergic system in animals due to particular sensitivity of DA neurons to energy impairment. In our study, the administration of malonate into the rat medial forebrain bundle (MFB) resulted in a 54% decrease in DA concentration and a 24–44% reduction of [^{3}H]GBR12,935 binding to the DAT 7 days after surgery (Lorenc-Koci et al. 2005b). TIQ administration (50 mg/kg), 4 h before malonate infusion and next once daily for 7 days, prevented the decrease in DA content and in [^{3}H]GBR12,935 binding to DAT. These results indicate that TIQ may act as a neuroprotective agent in the nigrostriatal dopaminergic system. However, the mechanisms by which TIQ exerts neuroprotective effect in this model are unknown. It seems that at least in part this effect may be attributed to antioxidant properties of GSH the level of which was significantly increased in the rat striatum after TIQ administration (Lorenc-Koci et al. 2001, 2005a). Furthermore, TIQ-mediated inhibition of MAO-dependent pathway of DA catabolism may also play an important role in the protection of striatal DA terminals from malonate destructive insults (Lorenc-Koci et al. 2000, 2005a).

There are also other experimental data that seem to indicate neuroprotective properties of TIQ. In particular, it was demonstrated in DAT cDNA transfected cell lines that TIQ prevented toxicity of MPP^+ and 2-$MeIQ^+$ ion (Storch et al. 2002). Moreover, in an abiotic system TIQ inhibited hydroxyl radical generation and in the rat SN it decreased production of free radicals (Antkiewicz-Michaluk et al. 2006; Lorenc-Koci et al. 2001). In mouse embryonic primary cultures, TIQ reduced glutamate toxicity measured as caspase-3 activity and lactate dehydrogenase release (Antkiewicz-Michaluk et al. 2006).

Recently, there has been a great controversy regarding a possible contribution of nitric oxide (NO) to the neurodegeneration of DA neurons in PD. Some studies have suggested that NO is a toxic molecule mediating death of DA neurons (LaVoie and Hastings 1999; Przedborski et al. 1996), whereas others have demonstrated its protective capacity against the oxidative stress (Kagan et al. 2001; Sharpe et al. 2003; Wink et al. 1996). Our study demonstrated that TIQ administered acutely and chronically (50 mg/kg i.p, two times per day for 14 days) significantly increased the tissue concentration of NO, measured as the level of nitrites, in the striatum, SN, and cortex, and in the whole rat brain (Lorenc-Koci et al. 2005a). Treatment with TIQ also increased the level of *S*-nitrosothiols, mainly *S*-nitrosoglutathione (GSNO) formed in the reaction between NO and GSH, in the whole rat brain and in the cortex though it reduced their level in the striatum. Blockade of the constitutive NO synthase by L-NAME in the presence of TIQ caused reduction in the GSH and

S-nitrosothiol levels (Lorenc-Koci et al. 2005a). The latter effect strongly suggested that NO affected biosynthesis of GSH and *S*-nitrosothiols in the rat brain. In the previous study (Lorenc-Koci et al. 2001), it was postulated that an increase in the GSH content after TIQ injection was a consequence of γ-GT inhibition and referred mainly to the extracellular pool of this peptide. However, the lack of elevation in the GSH content, by combined administration of L-NAME and TIQ, excludes such explanation. Hence, it has been postulated that TIQ acting via NO can increase GSH synthesis. However, a detailed mechanism of this modulation requires further experiments.

NO plays an important role as a cellular signaling molecule, vasodilator, anti-infectious agent, and as the most recently recognized, as an antioxidant (Kagan et al. 2001; Sharpe et al. 2003). A functional study demonstrated that TIQ at a dose of 50 mg/kg produced a strong and long-lasting (from 1 until 24 h after single dose) hypotensive effect, having decreased both systolic and diastolic blood pressure in rats (Michaluk et al. 2002). Authors of that paper have thought that this effect resulted from high affinity of TIQ for α_2-adrenergic receptors, but we suppose that both NO and GSH, the levels of which were markedly increased by TIQ treatment, could evoke this effect. Interestingly, soluble guanylyl cyclase, the target enzyme for NO-mediated signal transduction, is regulated by GSH, and under reduced oxygen tension, GSH- and NO-induced activation of this enzyme is additive (Niroomand et al. 1989, 1991). Since in TIQ treated rats the increase of NO was observed in the presence of high GSH concentration, it is likely that both these molecules act as antioxidants. Moreover, an increase of *S*-nitrosothiol level, mainly GSNO which is a 100-fold more potent antioxidant than GSH (Chiueh and Rauhala 1999), suggests that TIQ administration enhanced the antioxidant capacity of the rat brain.

The above-discussed results concerning the effect of TIQ on the levels of NO, GSH, and *S*-nitrosothiols seems to abrogate the hypothesis that TIQ may be a parkinsonism-inducing compound.

1.9 Conclusions

The experimental data assembled in the present review allow for a more precise characterization of the activity of the exogenous TIQ in the mammalian brain, especially in the nigrostriatal dopaminergic system. Based on these studies, the following conclusions may be drawn:

1. Exogenous TIQ indicated a high affinity for the brain tissue. Its concentration in the rat brain was several-fold higher than that in plasma both after acute and chronic treatment. In the nigrostriatal dopaminergic system, TIQ concentration after chronic treatment was twofold higher in the SN than that in the striatum. A half-life of TIQ in the rat brain was $t_{1/2}$ = 3 h 58 min while the respective value in the plasma was $t_{1/2}$ = 2 h 38 min.

2. Exogenous TIQ was metabolized to a minimal extent via 4-hydroxylation catalyzed in the rat liver by CYP isoenzymes belonging to CYP2D subfamily. Hence, this reaction in the liver has no influence on TIQ accumulation in the brain and on its elimination from the rat organism.
3. Exogenous TIQ was actively transported from the blood into the brain by OCT system, mainly by OCT3, and quickly eliminated from it by P-gp. Inhibition of P-gp activity slowed down TIQ elimination from the rat brain, suggesting that the accumulation of this compound in the brain, postulated previously to be a risk factor of PD, could be coupled rather with a genetic defect of P-gp than with that of CYP2D.
4. TIQ increased the level of the reduced GSH and GSH:GSSG ratio in the whole rat brain and in the dopaminergic structures what meant that the redox state of GSH/GSSG couple was shifted in a favor of the reduction reactions. In the reductive environment, there is no danger of excessive disulfide formation, so in such a condition TH cannot be inactivated by S-glutathionylation. This effect suggests that TIQ affecting the redox state of GSH/GSSG couple may increase the activity of TH and in this way it can modulate DA synthesis.
5. TIQ increased DA release in the striatum and SN which was directly confirmed using a microdialysis method and indirectly by the enhanced level of extracellular DA metabolite 3-MT.
6. TIQ inhibited the oxidative MAO-dependent DA catabolism and activated the COMT-dependent pathway. Such effects of TIQ on the course of both these reactions suggest that the compound in question may possess neuroprotective properties.
7. TIQ displaced [^{3}H] apomorphine from its binding sites within dopamine D_1 and D_2 receptors with effectiveness similar to DA and in a behavioral test inhibited the apomorphine-stimulated locomotor activity. The latter effect suggests that TIQ can attenuate dopaminergic neurotransmission at sites other than classical neuroleptics. Neuroleptic-like activity of TIQ could be responsible for some motor deficits observed in acutely and chronically TIQ-treated animals.
8. TIQ increased the antioxidant capacity of brain cells as it simultaneously enhanced the levels of GSH, NO, and *S*-nitrosothiols, mainly GSNO, and all these compounds possess neuroprotective properties. In the unilaterally malonate-lesioned rats, TIQ prevented the loss of DA and decline of [^{3}H]GBR12,935 binding to DAT. Moreover, in mouse embryonic primary cultures, TIQ reduced glutamate toxicity measured by caspase-3 activity.
9. Despite its structural similarity with MPTP, TIQ does not seem to be a toxic compound. Effects of TIQ on the synthesis, release, and catabolism of DA and on the binding of [^{3}H] apomorphine to dopamine D_1 and D_2 receptors suggest that this compound can modulate dopaminergic neurotransmission. Moreover, TIQ influence on GSH, NO, and *S*-nitrosothiol levels and its activity in the malonate and glutamate models of toxicity indicate that this compound can act not only as a modulator of dopaminergic neurotransmission but also as a neuroprotective agent.

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Chapter 2
Isoquinolines as Neurotoxins: Action and Molecular Mechanism

Agnieszka Wąsik and Lucyna Antkiewicz-Michaluk

Contents

Abstract Derivatives from the isoquinoline group were found in many plants, food as well as in the mammalian brain. The interest with these substances appeared about 20 years back, after the exploration of their chemical structures similar to the well-known exogenous neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Tetrahydroisoquinolines such as 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ) and 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) show the neurotoxic activity to the dopamine neurons and this way it has been proposed as endogenous factors leading risks to Parkinson's disease. In animals, research indicates that chronic administration of 1BnTIQ as well as salsolinol induced parkinsonian-like symptoms. Both compounds produce disturbances in the function of dopaminergic neurons, intensify oxidative stress, and inhibit mitochondrial complex I and/or II activity. In consequence, this mechanism of action leads to

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cell death via apoptosis. This review briefly describes the properties of 1BnTIQ and salsolinol in mammalian brain. This chapter presents the chemical structures of both compounds and possible pathways of their synthesis in the brain. A special focus was put on neurochemical effects of acute and chronic administration of 1BnTIQ and salsolinol on dopamine release as well as their metabolism in rat brain. Additionally, the effects of dopamine metabolism have been shown as a source of free radical generation in the brain.

Keywords 1-Benzyl-1,2,3,4-tetrahydroisoquinoline • Salsolinol • Rat brain • Oxidative stress • Neurotoxins • Dopamine metabolism • In vivo dopamine release • Parkinson's disease

Abbreviations

BBB	Blood–brain barrier
1BnTIQ	1-Benzyl-1,2,3,4-tetrahydroisoquinoline
COMT	Catechol-*O*-methyltransferase
CSF	Cerebrospinal fluid
DA	Dopamine
DAT	Dopamine transporter
DOPAC	3,4-Dihydroxyphenylacetic acid
H_2O_2	Hydrogen peroxide
HVA	Homovanilic acid
L-DOPA	3,4-Dihydroxy-L-phenylalanine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MAO	Enzyme monoamine oxidase
PEA	Phenylethylamine
PD	Parkinson's disease
ROS	Reactive oxygen species
TH	Tyrosine hydroxylase
TIQ	1,2,3,4-Tetrahydroisoquinoline

2.1 Introduction

Isoquinoline derivatives, e.g. tetrahydroisoquinolines, are widely distributed in the environment, being present in many plants and foods such as cheese, milk, red wine, bananas, etc. The exogenously administered tetrahydroisoquinolines easily cross the blood–brain barrier (BBB) and migrate into the brain, producing behavioral and biochemical effects in monoamine systems (Antkiewicz-Michaluk et al.

2000a, b, 2001; Kikuchi et al. 1991; Michaluk et al. 2002). These compounds belong to the isoquinoline group and their structure closely resembles the well-known exogenous toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The neurotoxic role of MPTP depends crucially on its metabolite MPP^+, formed in glial cells in a reaction catalyzed by the MAO_B enzyme. The assumption that tetrahydroisoquinolines may be neurotoxic is based on their ability to form tetrahydroisoquinoline ions, analogous to MPP^+ (Maruyama et al. 1997; Naoi et al. 1994, 1989a, b), in fact an experimental parkinsonism was induced by TIQ in monkeys (Nagatsu and Yoshida 1988) and by a salsolinol derivative in rats (Naoi et al. 1996). While MPTP acts rapidly and produces irreversible neurotoxic changes after a single injection and its effects are strictly limited to the nigrostriatal dopamine system (Burns et al. 1985), tetrahydroisoquinolines produce no immediate neurotoxic effects; after acute administration, they produce marginal biochemical effects. Furthermore, tetrahydroisoquinolines do not potentiate the action of dopamine receptor antagonists but very effectively counteract the action of dopamine receptor agonists. In addition, tetrahydroisoquinolines bind to the agonistic sites of dopamine receptors.

2.2 The Chemical Structure of the Endogenous Neurotoxins: 1-Benzyl-1,2,3,4-Tetrahydroisoquinoline and 1-Methyl-6,7-Dihydroxy-1,2,3,4-Tetrahydroisoquinoline (Salsolinol) (Fig. 2.1)

2.3 The Synthesis of 1BnTIQ and Salsolinol in the Brain

1BnTIQ can be formed in vivo by mammalian brain enzymes from PEA and phenylacetaldehyde (metabolite of PEA) generated by MAO-B.

The biosynthetic pathway of 1BnTIQ (Fig. 2.2).

In vivo 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol) can be formed in the mammalian brain by three different mechanisms: (1) via the

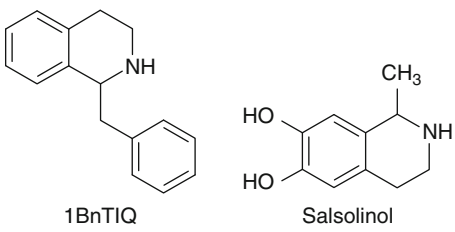

Fig. 2.1 Chemical structures of 1BnTIQ and salsolinol based on Naoi et al. 2004; Wąsik et al. 2009

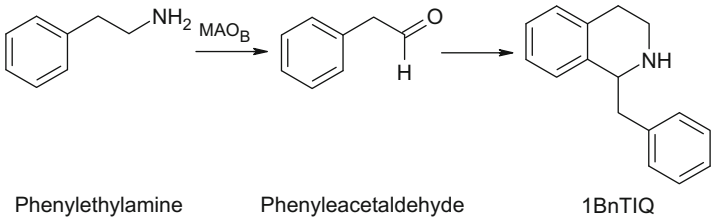

Fig. 2.2 Synthetic route of 1BnTIQ based on Kotake et al. 1995

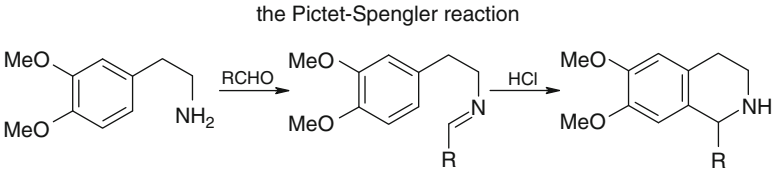

Fig. 2.3 The Pictet–Spengler reaction based on Whaley and Govindachari 1951

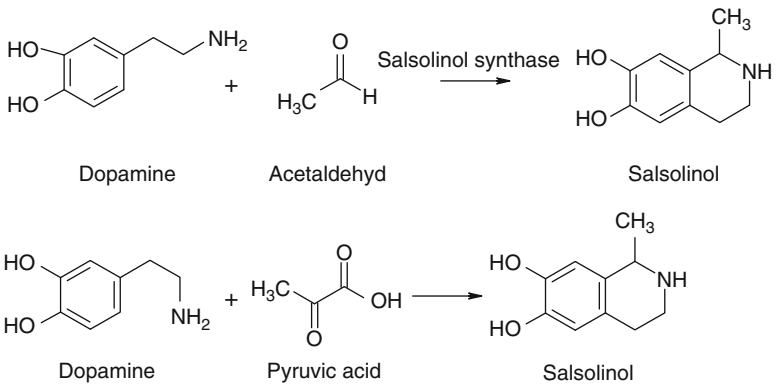

Fig. 2.4 Salsolinol synthesis based on Naoi et al. 2004

nonenzymatic Pictet–Spengler condensation of dopamine and aldehydes producing salsolinol in two racemic isomers (*R* or *S*); (2) by the nonenzymatic condensation of dopamine and pyruvate yielding 1-carboxyl-tetrahydroisoquinoline, followed by decarboxylation and reduction, which produce (*R*)-salsolinol; (3) by the selective synthesis of (*R*)-salsolinol from dopamine and acetaldehyde.

The original Pictet–Spengler reaction was a reaction of β-phenylethylamine with the dimethyl acetal of formaldehyde and hydrochloric acid, which yielded tetrahydroisoquinoline (Figs. 2.3 and 2.4).

2.4 The Oxidation of Dopamine

There are many natural sources of oxidative stress e.g., environmental toxins (herbicides, pesticides, heavy metals), heat shock, UV radiation, and inflammation. Reactive oxygen species (ROS) are products of normal cellular metabolism. Also, dopamine can generate ROS via enzymatic and nonenzymatic pathways (Cohen et al. 1997; Berman and Hastings 1999; Gluck et al. 2002). During a dopamine catabolism process, ROS are formed which are very dangerous to living cells. A high concentration of ROS leads to damage to a number of biomolecules, such as DNA, proteins, and lipids. In consequence, cell death is induced via apoptosis. Complexes I, II, and III of the mitochondrial respiration, pyruvate dehydrogenase, and α-ketoglutarate complexes are highly sensitive to the blocked effect of ROS (Vinogradov et al. 1976; Bunik et al. 1990; Bulteau et al. 2003; Bunik 2003). Gluck et al. (2002) reported that dopamine at low concentrations inhibited mitochondrial respiration, predominately by a MAO-dependent mechanism involving H_2O_2 and downstream hydroxyl radical formation. The production of superoxide anion occurs mostly within cell mitochondria (Cadenas and Sies 1998). In neurons, dopamine is nonenzymatically oxidized by the molecular oxygen to form hydrogen peroxide (H_2O_2) and the corresponding *O*-quinone (Oq). Then, this Oq undergoes intramolecular cyclization which is immediately followed by a cascade of oxidative reactions resulting in the formation of neuromelanin – a black pigment characteristic of dopaminergic neurons (Graham 1978; Graham et al. 1978). Additionally, dopamine can also be enzymatically deaminated by monoamine oxidase (MAO) to form H_2O_2 and 3,4-dihydroxyphenylacetaldehyde. This process is shown by the following formula:

$$\mathrm{DA} + \mathrm{O_2} + \mathrm{H_2O} \xrightarrow{\mathrm{MAO_B}} \mathrm{DOPAC} + \mathrm{NH_3} + \mathrm{H_2O_2}$$

Subsequently, 3,4-dihydroxyphenylacetaldehyde is oxidized by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid (DOPAC), which is methylated by catechol-*O*-methyltransferase (COMT) to yield homovanilic acid (HVA), which is a final dopamine metabolite. Therefore, both autoxidation and MAO-mediated metabolism of dopamine lead to the production of H_2O_2. This compound can be subject to the Fenton reaction, which consists in reducing H_2O_2 in the presence of ferrous iron (Fe^{2+}). Further, in consequence come into the being the hydroxyl radical ($^{\bullet}OH$), which is considered the most damaging free radical to living cells.

$$\mathrm{Fe^{2+}} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe^{3+}} + \mathrm{OH^-} + {}^{\bullet}\mathrm{OH}$$

The formation of a free radical during both the biosynthesis and the turnover of dopamine leads to a loss of many dopaminergic neurons (Fornstedt et al. 1990). It is evident that the subsequent excessive autoxidation and catabolism of dopamine are involved in the development of many neurodegenerative and age-related disorders (e.g., Parkinson's disease). Furthermore, these phenomena are enhanced by the presence of neuromelanin in dopaminergic neurons due to its reported ability to

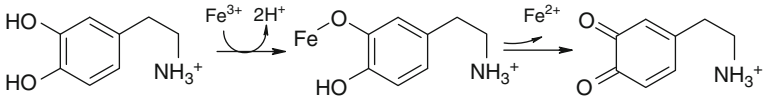

Fig. 2.5 Dopamine autoxidation based on Hermida-Ameijeiras et al. 2004

accumulate iron (Enoch et al. 1994); consequently, neuromelanin can act by promoting the Fenton reaction. Hermida-Ameijeiras et al. (2004) reported that the continuous production of •OH during the dopamine incubation with mitochondrial preparations obtained from rat brain was maintained under the physiological conditions of pH and temperature. That production was reduced when MAO activity was blocked by the preincubation of mitochondrial preparations with the MAO inhibitor, pargyline. Thus, on the one hand the dopamine protects against both the hazardous Fenton reaction and the propagation of lipid peroxidation but, on the other, it generates •OH and promotes protein oxidation. Furthermore, these properties are differently enhanced by the presence of Fe^{2+} and Fe^{3+}. The hydroxyl ion OH^-, also produced in the Fenton reaction, is considerably less toxic (10^{14}) than the hydroxyl radical. Iron plays an important role in this reaction. It may originate from neuromelanin or ferritin. It should be emphasized that in the course of Parkinson's disease, neuromelanin-containing dopaminergic neurons are those which die, which suggests that Fe^{2+}, essential for the Fenton reaction to occur, can be released from this compound by unknown toxic factors (Antkiewicz-Michaluk 2002; Ben-Shachar et al. 1991a, b). Thus, dopamine metabolism leads to the formation of the toxic hydroxyl radical, which poses a serious threat to nervous cells causing their damage and death in the process of apoptosis.

The process of dopamine autoxidation in the presence of Fe^{3+} is shown by the following formula (Fig. 2.5).

2.5 The Effect of Acute and Chronic Treatment with 1BnTIQ and Salsolinol on Dopamine Metabolism in Rat Brain

1-Benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ) is an endogenous neurotoxin which has been proposed as one of the etiological factors of idiopathic Parkinson's disease (PD) (Kotake et al. 1995). The level of 1BnTIQ in the CSF of patients with idiopathic PD was found to be three times higher than that in CSF of neurological control subjects (Kotake et al. 1995). Chronic administration of 1BnTIQ induced parkinsonian-like symptoms in rodents and primates (Kotake et al. 1995; 1996). In vitro studies showed that 1BnTIQ is toxic to human SH-SY5Y neuroblastoma cells and cultured primary neurons (Kotake et al. 2003; Shavali and Ebadi 2003; Shavali et al. 2004). Some evidence demonstrated that 1BnTIQ dose-dependently elevated

the level of the pro-apoptotic protein Bax and decreased the concentration of the anti-apoptotic protein Bcl-xL. Additionally, 1BnTIQ produced an increase in the formation of active caspase-3 protein fragments (Shavali and Ebadi 2003). 1BnTIQ induced cell death via apoptosis. A morphological analysis of SH-SY5Y cells treated with 1BnTIQ showed nuclear defects and the presence of apoptotic-like bodies and nuclear fragments (Shavali et al. 2004). Dopaminergic cells deteriorated and slowly died, their number being gradually reduced. The neurotoxicity of 1BnTIQ was correlated with the overall exposure (concentration multiplied by time of exposure). The prolonged exposure of dopaminergic neurons to a low concentration of 1BnTIQ initially induced a decrease in the dopamine level, after which the shrinkage of the cell body led to cell death (Kotake et al. 2003). Different TIQ derivatives inhibited mitochondrial respiration and electron transfer complexes. 1BnTIQ was found to be a more potent inhibitor than MPTP and MPP+ (Morikawa et al. 1996, 1998). 1BnTIQ also blocked the dopamine transporter (DAT) leading to inhibition of dopamine uptake. Okada et al. (1998) reported that 1BnTIQ can be taken up via DAT into dopaminergic neurons similarly to MPP^+ in vivo. Otherwise, those agents can only bind to the DAT like cocaine. However, since salsolinol (structurally similar to THP) seems to be taken up into rat striatal slices (Hirata et al. 1990) and PC 12h cells (Maruyama et al. 1993) 1BnTIQ can also be accumulated in DAT-HEK. 1BnTIQ, which is synthesized endogenously in the body and/or is obtained exogenously in the diet, can be taken up by neurons via DAT; furthermore, it accumulates in dopaminergic neurons and exerts some pathological effects leading to parkinsonism, and it disturbs the efficacy of L-DOPA chemotherapy in parkinsonian patients. Kotake et al. (2003) showed that the exposure to 1BnTIQ for 24 h or 7 days caused a dose-dependent decrease in dopamine content in mesencephalic slices. Kohta et al. (2010) found that 1BnTIQ bound to tubulin β in midbrain neurons and reduced the formation of high-molecular-weight polyubiquitinated tubulin β. The latter findings suggest that 1BnTIQ may impair tubulin β ubiquitination, similarly to mutant parkin in AR-JP. Even low concentrations of 1BnTIQ can decrease the polyubiquitination of tubulin β if present for a long time (Kohta et al. 2010). The overexpression of tubulin β is toxic (Burke et al. 1989) and causes disturbances in the functioning of dopaminergic neurons. 1BnTIQ acts by inhibiting the enzymes involved in dopamine biosynthesis. Ex vivo biochemical studies showed that a single dose of 1BnTIQ (50 mg/kg) produced a dramatic fall in the dopamine level in rat brain (approx. 40%) and increased the concentration of its metabolites, DOPAC and HVA. Additionally, 1BnTIQ markedly reduced the level of extraneuronal dopamine metabolite, 3-MT. 1BnTIQ evoked strong (nearly threefold) activation of the oxidative MAO-dependent catabolic pathway (Wąsik et al. 2009). Dopamine oxidation is directly connected with the production of free radicals, oxidative stress, as well as with cell death and neurodegeneration (Schapira et al. 1990; Adams and Odunze 1991; Miller et al. 1996; Chan 1998; Dykens 1999). At the same time, 1BnTIQ significantly inhibits the COMT-dependent O-methylation pathway. Striatum and nucleus accumbens represent brain regions where the depression of dopamine produced by 1BnTIQ is most powerfully expressed, this effect being specific to dopaminergic neurons. The biochemical effects of the chronic

administration of 1BnTIQ are considerably weaker. This pattern of changes suggests that during chronic 1BnTIQ administration some tolerance to its dopamine-depressing effect develops, while the impairment of dopamine synthesis ensues (Wąsik et al. 2009). After chronic (14 doses) 1BnTIQ administration the decrease in dopamine level was weaker (approx. 20%). However, in the mixed group in which rats received L-DOPA with the last dose of 1BnTIQ, the effects of L-DOPA were significantly reduced. Such an effect was observed 2 h after the last 1BnTIQ injection, as well as after its 24-h withdrawal. Hence, dopamine production was disturbed after chronic 1BnTIQ administration, the effect being long lasting (Antkiewicz-Michaluk et al. 2010).

It is common by known that enantiomer (*R*)-salsolinol is synthesized in human and mammalian brain, whereas enantiomer (*S*) penetrates into the organism with foods. A low concentration of salsolinol was detected in normal human cerebrospinal fluid (Moser and Kompf 1992), brain, and urine (Dostert et al. 1989). In contrast, both parkinsonian patients treated with L-DOPA and chronic alcoholics showed a significant elevation in the concentration of salsolinol in CSF and urine (Cohen and Collins 1970; Collins et al. 1979; Moser and Kompf 1992; Sandler et al. 1973). Salsolinol is a dopamine metabolite and its toxicity is closely connected with catecholaminergic nerve terminals. Salsolinol is structurally similar to MPTP which produces a parkinsonian-like syndrome in human and nonhuman primates. It was suggested that under special conditions salsolinol may act as a false neurotransmitter, causing – among other effects – neurodegeneration. It was found that tetrahydroisoquinoline may produce parkinsonism-like symptoms in primates (Nagatsu and Yoshida 1988). Salsolinol acts as inhibitor both of tyrosine hydroxylase (TH) and MAO. Patsenka and Antkiewicz-Michaluk (2004) have reported that different TIQs inhibited MAO activity in a dose-dependent manner. Salsolinol inhibited MAO_A activity most effectively in rat frontal cortex, and less efficiently in other rat and mouse brain structures. Moreover, from different TIQs only salsolinol was effective as an inhibitor of TH activity. This compound is regarded as an inhibitor of catecholamine uptake in rat brain synaptosomes and it causes the release of catecholamines stored in rat brain (Heikkila et al. 1971). Storch et al. (2000) concluded that salsolinol was toxic to dopaminergic neuroblastoma SH-SY5Y cells by blocking the cellular energy supply via inhibition of mitochondrial complex II activity. The latter authors found that incubation of human SH-SY5Y dopaminergic neuroblastoma cells with salsolinol resulted in a rapid, dose- and time-dependent decrease in the intracellular level of ATP and ATP/ADP ratio of intact cells. In vitro studies showed that salsolinol induced specific changes in cellular energy metabolism, similar to those caused by MPP^+, which consistently preceded cell death (Storch et al. 2000). As reported by Morikawa et al. (1998) salsolinol inhibited mitochondrial complex II activity. It caused a rapid loss of intracellular ATP and maximal turnover of glycolysis without compensating fast energy depletion. Additionally, the blockade of complex II did not change the level of NADH. Selective binding of salsolinol was confirmed not only in dopaminergic structures such as e.g., the striatum, but also in the pituitary gland, cortex, and hypothalamus (Homicsko et al. 2003). Salsolinol also inhibited vesicular monoamine transporters in dopaminergic terminals.

The latter findings suggest that salsolinol may regulate the function of dopamine neurons as a neurotransmitter and may act as a mediator in the neuroendocrine system, through its specific binding sites and via intervention in the dopamine system (Naoi et al. 2004). Salsolinol antagonized behavioral action of L-DOPA and apomorphine, a dopamine agonist (Ginos and Doroski 1979; Antkiewicz-Michaluk et al. 2000a, b). Binding studies demonstrated that salsolinol displaced [^{3}H]apomorphine, but not dopamine D_1 ([^{3}H]SCH23,390) and D_2 ([^{3}H]spiperone) receptor antagonists, from their binding sites, its effectiveness being comparable to that of dopamine (Antkiewicz-Michaluk et al. 2000a, b). The above data suggest that salsolinol may suppress dopaminergic transmission by acting on the agonistic sites of dopaminergic receptors, which are different from neuroleptic binding sites. Salsolinol showed an antidopaminergic profile since it induced only a weak effect on spontaneous locomotor activity; moreover, it produced effective antagonism to behavioral and biochemical effects of apomorphine and induced muscle rigidity (Antkiewicz-Michaluk et al. 2000a, b; Lorenc-Koci et al. 2000; Vetulani et al. 2001). Ex vivo biochemical studies demonstrated that a single dose of salsolinol (100 mg/kg) produced no changes in dopamine concentration as well as its metabolites in different rat brain structures. On the other hand, administration of salsolinol jointly with L-DOPA enhanced its effect. In fact, the level of dopamine and all its metabolites was significantly higher compared to a group treated with L-DOPA (data not shown). Chronic (14 doses) salsolinol administration did not produce any changes in dopamine concentration and in the level of its metabolites. However, in a mixed group of rats which were given, the last dose of salsolinol jointly with L-DOPA, the effect of L-DOPA was significantly reduced. Similar to experiment with 1BnTIQ, the latter effect was observed 2 h after the last salsolinol injection as well as after its 24-h withdrawal. Hence, it has been demonstrated that chronic injection of salsolinol produces long-lasting disturbances in dopamine production in the brain (Antkiewicz-Michaluk et al. 2010).

2.6 The Effect of 1BnTIQ and Salsolinol on In Vivo Dopamine Release in Rat Striatum

An in vivo microdialysis study demonstrated that 1BnTIQ given systemic markedly reduced dopamine release into the synaptic cleft of freely moving rats, and produced a long-lasting decrease in extracellular dopamine in rat striatum (about 30%). In contrast, the concentration of all the dopamine metabolites was significantly elevated after acute 1BnTIQ administration (approx. 100%) (Wąsik et al. 2009). The above findings suggest that 1BnTIQ may show injury properties to vesicular transporter in dopaminergic neurons, leading to a pathological release of dopamine into the cytosol. In contrast, an acute dose of salsolinol produced only a slight reduction of dopamine level. The latter findings indicate that both acute and repeated administration of 1BnTIQ results in the development of abnormalities in the function of dopamine neurons (Wąsik et al. 2009). Such disturbances are particularly observed in

animals treated with 1BnTIQ concomitant with L-DOPA, because chronic administration of 1BnTIQ in contrast to its acute injection significantly reduces the increase in dopamine release, produced by L-DOPA; furthermore, it completely antagonizes the L-DOPA-induced rise in DOPAC and 3-MT levels in rat striatum (Antkiewicz-Michaluk et al. 2010). In the light of all these data we suggest that therapy with L-DOPA may not be efficient in PD patients with an elevated endogenous 1BnTIQ level in the brain.

2.7 Conclusions

It is reported that both isoquinoline derivatives (1BnTIQ and salsolinol) are endogenous neurotoxins synthesized in human and mammalian brain. The action of both these compounds is closely connected with dopaminergic neurons. Their exogenous administration elevates dopamine oxidation which leads to an increase in the formation of ROS in dopaminergic neurons. In consequence, these substances inhibit the mitochondrial complex I or/and II and induce cell death via apoptosis. Chronic administration of 1BnTIQ as well as salsolinol produced parkinsonian-like symptoms both in rodents and rhesus. Both acute and chronic administration of 1BnTIQ and salsolinol leads to the development of the abnormalities in the functioning of dopamine neurons. The accumulated evidence suggests that isoquinoline derivatives may be one of the etiological factors of idiopathic PD and additionally a therapy with L-DOPA may not be efficient in PD patients with a high endogenous level of 1BnTIQ and/or salsolinol in the brain.

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Chapter 3
1-Methyl-1,2,3,4-Tetrahydroisoquinoline: A Potent Neuroprotecting Agent

Jerzy Vetulani and Lucyna Antkiewicz-Michaluk

Contents

Abstract 1-Methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ), unlike several other tetrahydroisoquinolines, displays neuroprotective properties. To elucidate this action we compared the effects of 1MeTIQ with 1,2,3,4-tetrahydroisoquinoline (TIQ), a compound sharing many activities with 1MeTIQ (e.g., reducing free radicals formed during dopamine catabolism) but offering no clear neuroprotection. We found that the compounds similarly inhibit free radical generation in an abiotic system, as well as indices of neurotoxicity, caspase-3 activity, and lactate dehydrogenase release

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induced by glutamate in mouse embryonic primary cell cultures. 1MeTIQ also prevents the glutamate-induced cell death and $^{45}Ca^{2+}$ influx, whereas TIQ did not. In vivo microdialysis study has shown that 1MeTIQ prevents kainate-induced release of excitatory amino acids from the rat frontal cortex. Additionally, 1MeTIQ protects against rotenone-induced mortality, oxidative stress as well as dopaminergic neurodegeneration in the extrapyramidal structures produced by intracerebral injection of rotenone. The results suggest that 1MeTIQ offers a unique and complex mechanism of neuroprotection in which free radicals scavenging properties and inhibition of glutamate-induced excitotoxicity may play a very important role, and indicates the potential of 1MeTIQ as a therapeutic agent in various neurodegenerative illnesses of the central nervous system.

Keywords 1-Methyl-1,2,3,4-tetrahydroisoquinoline • Rat brain • Dopamine metabolism • MAO inhibition • Rotenone • Glutamate • Oxidative stress • Free radicals • Neuroprotection • Parkinson's disease

3.1 Introduction

Tetrahydroisoquinolines, compounds of which 1-methyl-1,2,3,4-tetrahydroquinoline (1MeTIQ) is a representative, form an interesting group among several classes of brain chemicals. Numerous tertrahydroisoqunoline compounds were found to be present in the brain tissue (Zarranz de Ysern and Ordonez 1981; Niwa et al. 1987), some of them being endogenous, others taken up with foods, still others being at the same time endo- and exogenous (Niwa et al. 1989; Deng et al. 1997; Duncan and Smythe 1982). Although tetrahydroisoquinolines are present in normal brains only in minute quantities (Weiner 1981), they may be generated or accumulated in critical sites of the brain, for example, in the vicinity of dopamine neurons, and exerting various biological activities may importantly interfere with specific brain structures and functions. The compounds of this class have some biological activities essentially similar [e.g., antagonism of receptors and/or attenuation of neurotransmitter release in dopaminergic (Antkiewicz-Michaluk et al. 2000, 2001; Ginos and Doroski 1979)] and glutamatergic structures (Lorenc-Koci et al. 2009; Ueda et al. 1999; Ludwig et al. 2006), while they differ profoundly among themselves in their neurotoxicity. Some of them, for example, N-methyl, 1,2,3,4-tetrahydroisoquinoline (N-MeTIQ) are highly neurotoxic (Maruyama et al. 1993), others, like 1MeTIQ, possess neuroprotective properties (Tasaki et al. 1991). The neuroprotective action of some of them may be related to their inhibitory action on monoamine oxidase activity and/or on their action as free radical scavengers (Maruyama et al. 1995). Some derivatives were even reported to protect against NMDA-induced neural damage (Ohkubo et al. 1996).

Nevertheless, the fact that neurotoxic tetrahydroisoquinolines are preserved in the brain in the course of evolution together with nonneurotoxic species suggests that both groups play a physiological role in the brain, and we proposed that they may be natural regulators of the dopaminergic system, preventing its excessive phasic activity without affecting the tonic one (Vetulani et al. 2003a).

Even before their discovery in the brain, tetrahydroisoquinolines aroused the interest of pharmacologists. The early studies on 1,2,3,4-tetrahydroisoquinoline (TIQ) and its derivatives revealed their neuroleptic-like properties (Ginos and Doroski 1979) and our more recent results suggest that TIQ and its derivatives are antagonists of agonistic conformation of dopamine D2 receptor (Antkiewicz-Michaluk et al. 2000; Vetulani et al. 2001; Vetulani et al. 2003a). This explains why TIQ and its congeners effectively block dopaminergic stimulation without affecting much the basal locomotor activity. The finding that TIQ potentiated morphine-induced running fit in mice (Vetulani et al. 2001) corroborated the assumption that tetrahydroisoquinolines do not block dopamine receptors in a manner similar to that of classical neuroleptics and focused our attention on a possible interaction of these compounds with other actions of morphine. This line of research led to the discovery of potentiation of morphine and oxotremorine-induced analgesia by simple tetrahydroisoquinolines, including 1MeTIQ (Vetulani et al. 2003b), prevention of morphine abstinence syndrome (Wasik et al. 2007) and attenuation of cocaine sensitization (Wasik et al. 2010) and prevention of cocaine-induced relapse to self-administration (Filip et al. 2007).

Pharmacologically tetrahydroisoquinolines aroused also an interest as potential NMDA receptor antagonists (Ortwine et al. 1992). Some of them were described as effective antagonists of the PCP site (Rogawski et al. 1989). However, most of tetrahydroisoquinolines, do not substitute for PCP (Nicholson and Balster 2003).

Among several endogenous tetrahydroisoquinolines 1MeTIQ has a special position, as very early it was described in the brain (Kohno et al. 1986; Makino et al. 1990; Niwa et al. 1987; Ohta et al. 1987), and shortly after recognized as a potential antiparkinsonian agent on the base of reversal of bradykinesia induced by MPTP, TIQ, or 1BnTIQ (Tasaki et al. 1991; Kotake et al. 1995).

3.2 Synthesis of 1MeTIQ in the Brain

1-MeTIQ was identified in normal rat brains in 1986 (Kohno et al. 1986), and subsequently found to be present in foods rich in 2-phenylethylamine, from which it may enter the brain (Makino et al. 1988), but it is also synthesized in the brain (Niwa et al. 1990; Tasaki et al. 1993). Its cerebral concentration in normal rat brains was recently determined as 3.4 ± 1.5 (0.50 ± 0.22 ng/g), exceeding threefold to fivefold the concentrations of other simple tetrahydroisoquinolines (Inoue et al. 2008). Having an asymmetric carbon atom, 1MeTIQ may appearing the form of R and S stereoisomers, and the product found in brain and in foods is a racemate (Makino et al. 1990) and the stereoisomers differ in some respect in their biological action (Abe et al. 2001).

1MeTIQ is enzymatically formed in the brain from 2-phenethylamine and pyruvate. The enzyme involved, 1MeTIQase, was localized in the mitochondrial–synaptosomal fraction of rat brain, isolated and purified. 1MeTIQase may be important in the pathogenesis of Parkinson's disease. (Yamakawa and Ohta 1997).

Its activity is spread throughout the brain, the highest activity being observed in the dopaminergic areas that are implicated in the etiology of Parkinson's disease (striatum and substantia nigra) and in the cortex. During aging the activity of 1-MeTIQse falls (by approximately 50%) in the areas of its highest activity (Absi et al. 2002).

Most of the studies on 1MeTIQ were carried out on rodents' brains, but the results on monkeys, showing that the regional distribution of contents of 1MeTIQ, and other simple tetrahydroisoquinolines and activity of 1MeTIQase presented a similar pattern of distribution (Yamakawa et al. 1999).

1MeTIQ synthesis is inhibited by agents that induce experimental Parkinsonism (Tasaki et al. 1991; Yamakawa and Ohta 1999; Igarashi et al. 1999), its concentration in the substantia nigra declines in aged rats (up to 50%) and in Parkinsonian patients (Ayala et al. 1994). All those data indicate that the change of the 1-MeTIQ content in the brain plays an important role in the pathogenesis of toxin-induced Parkinsonism, and that the degeneration of the dopaminergic neurons may proceed as a result of the loss of neuroprotection offered by 1-MeTIQ.

When injected systematically 1MeTIQ easily passes through the blood–brain barrier, and accumulates in the brain (Kikuchi et al. 1991). As mentioned earlier, 1MeTIQ may appear in stereoisomeric forms, R and S, which have different biological properties. It appears that (R)-1-MeTIQ, and not (S)-enantiomer, plays a crucial role as a neuroprotective antiparkinsonian agent (Abe et al. 2001).

3.3 Scavenging of Free Radicals by 1MeTIQ

To find out whether 1MeTIQ may protect against oxidative stress we investigated its capacity to inhibit hydroxyl radical generation in vitro. Oxidative stress leads to the production of reactive oxygen species (Harman 1981), such as superoxide anion radical (O2-) and hydroxyl radical (OH) that are known to damage all cellular biomacromolecules (lipids, sugars, proteins, and polynucleotides), and this damage can lead to equally damaging secondary products (Sayre et al. 2008), Owing to that the oxidative stress was since long regarded as a universal mechanism of inducing cell death (Dykens 1999). In the brain the main source of toxic hydroxyl radical formation and H_2O_2 generation is the oxidative deamination of monoamines by the action of MAO (for review see Singer and Ramsay 1995). Thus, the excessive dopamine catabolism by MAO augments the formation of free radicals in the brain. After having demonstrated that 1MeTIQ and other simple tetrahydroisoquinolines inhibit MAOA and MAOB activities from mouse and rat brains, thus having potentially antioxidant properties, (Patsenka and Antkiewicz-Michaluk 2004), a direct study on the free radical scavenging capacity of 1MeTIQ was carried out. To measure the antioxidant capacity of free radical scavengers the Fenton reaction is suitable, as it generates the hydroxyl radicals in a manner similar to their in vivo generation. In this "inorganic" model 1MeTIQ inhibited the free radical formation and completely abolished the dopamine-enhanced efficiency of Fenton reaction (Antkiewicz-Michaluk et al. 2006).

Those results demonstrate that regardless of its interaction with biological structures, 1MeTIQ has intrinsic antioxidant properties.

3.4 Neuroprotection of 1MeTIQ in Relation to Dopaminergic Mechanisms

3.4.1 Effect of Acute and Chronic 1MeTIQ Administration on Dopamine Metabolism in the Brain: Inhibition of MAO-Dependent Dopamine Oxidation Pathway In Vivo

Several tetrahydroisoquinolines and their congeners interfere with monoamine oxidases (MAO). Many isoquinolines were found to selectively inhibit either MAO A or (less frequently) MAO B. Stereoselective competitive inhibition of MAO A was found with the (R) enantiomer of all the stereoisomers tested (Bembenek et al. 1990). In those early experiments 1MeTIQ was not tested, but 2-Me-1,2,3,4-tetrahydroisoquinoline was much more potent than unsubstituted 1,2,3,4-tetrahydroisoquinoline, and both preferably inhibited MAOB. Later on the inhibitory effect of 1MeTIQ and 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline were found to inhibit the activity of MAOB, indicating that they may be neuroprotective agents in the brain, and their involvement in the pathogenesis of Parkinson's disease was discussed (Naoi and Maruyama 1993). Afterward Thull et al. (1995) investigated 45 isoquinoline derivatives and found most of them reversible inhibitors of MAOA and B, often selective to the A form. Their studies brought to the forefront the question of the physiological significance of endogenous MAO inhibitors and suggested a role for endogenous tetrahydroisoquinolines in the control of neurotransmitter function, and prevention of neurotoxicity related to MAO activity in the brain. More recently the effectiveness of 1MeTIQ as an inhibitor of MAOA and MAOB was compared with that of 1,2,3,4-tetrahydroisocarboline, and 1MeTIQ was found roughly equipotent with its unsubstituted congener as MAOA inhibitor, while less effective in regard to MAOB (Patsenka and Antkiewicz-Michaluk 2004).

The data from ex vivo neurochemical experiments have shown stereospecificity of 1MeTIQ enantiomers in respect of their effects on dopamine catabolism. While both the enantiomers increased the concentrations of dopamine and its extraneuronal metabolite, 3-MT in rat striatum, they differently affected dopamine catabolism. Thus, (R)-1MeTIQ significantly increased both the level of the final DA metabolite, HVA (by about 70%), and the rate of DA metabolism (by 50%), while (S)-1MeTIQ significantly depressed the DOPAC and HVA levels (by 60 and 40%, respectively), and attenuated the rate of DA metabolism (by about 60%) (Antkiewicz-Michaluk et al. 2011). These data suggest that the (S)- enantiomer may offer greater protection against neurotoxicity.

3.4.2 Lack of Noxious Effect of 1MeTIQ Administration on Dopamine Neurons

As tetrahydroisoquinolines show structural resemblance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a potent neurotoxin capable of producing persistent parkinsonism in humans (Langston et al. 1983) which could be employed for producing experimental Parkinson's disease in laboratory animals (Jenner and Marsden 1986), initially they all were assumed to cause damage to dopamine neurons In fact the early studies reported that they generally are neurodegenerating agents (Suzuki et al. 1990), the most neurotoxic being 1,2,3,4-tetrahydroisoquinoline (TIQ), 1-benzyl-TIQ, and (R)-1,2-dimethyl-5,6-dihydroxy-TIQ (R)-N-methyl-salsolinol (Nagatsu 1997). This finding contrasted with an earlier report which found no neurotoxicity of TIQ on nigrostriatal dopamine neurons (Perry et al. 1988). The most recent studies, in which the actions of 1MeTIQ and TIQ were directly compared, suggested that TIQ in fact produces some damage to dopaminergic neurons, which is reflected by a mild but significant decrease in the striatal dopamine concentration in rats that were chronically administered with TIQ. In contrast, 1MeTIQ never caused a decline in the dopamine levels in brain structures, although both tetrahydroisoquinolines similarly affected dopamine catabolism (Antkiewicz-Michaluk et al. 2000).

3.4.3 Action of 1MeTIQ on Dopamine Nerve Terminals

Interaction with presynaptic dopamine receptors was investigated by studying 1MeTIQ-induced displacement of dopamine receptor ligands from their binding sites. In general tetrahydroisoquinolines do not displace antagonistic ligands bound to dopamine D2 receptors (Antkiewicz-Michaluk et al. 2000; Vetulani et al. 2003a), the exception being [^{11}C]raclopride.

Depression of binding of [^{11}C]raclopride may be interpreted as the sign of increased synaptic dopamine concentrations, which competes with raclopride at D2 receptor sites (Laruelle 2000). Tetrahydroisoquinolines were shown to displace [^{11}C]raclopride, and (S)-enantiomer of 1MeTIQ and TIQ were most potent in this respect. The effect of TIQ continued for 7 h and was followed by an increase until 10 days after the single and subchronic administration of TIQ. These findings suggest that TIQ analogs profoundly stimulated dopamine release which resulted in the competitive inhibition of the binding of [^{11}C]raclopride to dopamine D2 receptors, but did not induce degeneration of the receptors (Ishiwata et al. 2001).

Another D2 receptor ligand displaced by TIQ was the D2 receptor agonist, [^{3}H] apomorphine (Antkiewicz-Michaluk et al. 2000; Vetulani et al. 2003a). In contrast to antagonists, an agonist radioligand binds preferentially to the high-affinity state and is expected to have greater sensitivity to DA, which is the endogenous agonist. Studies on the effects of amphetamine on displacement of D2 receptor ligands, an agonist [^{11}C](R)-2-CH_3O-N-n-propylnorapomorphine and agonist, [^{11}C]raclopride demonstrated that the agonist is more sensitive than antagonist to

displacement by endogenous dopamine (Seneca et al. 2006). Thus, the experiments with [^{3}H]apomorphine displacement confirm that tetrahydroisoquinoliones may release dopamine from dopaminergic terminals. However, owing to their MAO-inhibiting properties they do not cause neurodegeneration of dopaminergic neurons.

In stereoselective study, it has been documented that the neuroprotective action of 1MeTIQ against neurotoxicity is closely related with the presence of (R)-1MeTIQ enantiomer as well as raceme, the mixture of (R)- and (S)-enantiomers (Abe et al. 2001). In biochemical studies assessing activity of 1-MeTIQ synthesizing enzyme (1MeTIQ-ase), it has been reported that the parkinsonism-inducing substances such as MPTP and β-carbolines considerably inhibited the activity of 1MeTIQ-ase (Yamakawa and Ohta 1999). It is also well established by behavioral and biochemical study that (R,S)-1MeTIQ demonstrates neuroprotective activity by antagonizing the behavioral and biochemical effects of dopaminergic neurodegeneration induced by numerous experimental neurotoxins such as MPTP, 1BnTIQ, rotenone (Antkiewicz-Michaluk et al. 2003, 2004; Kotake et al. 1995, 2005; Tasaki et al. 1991).

3.5 Neuroprotection of 1MeTIQ Against Rotenone, a Dopaminergic Neurotoxin

It was recently shown by Betarbet et al. (2000) that rotenone, an environmental toxin may be used to produce a more realistic MPP$^+$ animal model of Parkinson's disease. Rotenone, a natural compound, is a classical, lipophilic inhibitor of mitochondrial complex I (Gutman et al. 1970; Horgan et al. 1968), and selectively toxic to dopaminergic neurons (Marey-Semper et al. 1993). Injected directly into brain structures, rotenone acts similarly to MPTP (Heikkila et al. 1985).

Rotenone is the only neurotoxin known today that induces the formation of Lewy bodies, which are the most characteristic histopathological feature of Parkinson's disease (Betarbet et al. 2000). A defect of mitochondrial function due to complex I inhibition was postulated to be the cause of rotenone-induced neurodegeneration (Jenner 2001; Greenamyre et al. 2001). Rotenone also causes dopamine release, as evidenced by microdialysis and neurochemical data (Santiago et al. 1995; Thiffault et al. 2000), and this may also contribute to the degeneration of dopaminergic neurons. We have found that rotenone administered peripherally in a single dose did not produce evident behavioral or biochemical effects. In contrast, repeated administration of rotenone (12 mg/kg s.c.) causing abnormalities in general behavior, produced considerable mortality and dramatic increases in dopamine metabolism, which may be ascribed to an increase in the oxidative pathway, and strongly depressed the concentration of the extracellular dopamine metabolite, 3-MT. These behavioral and biochemical changes were effectively counteracted by administration of 1MeTIQ before each dose of rotenone (Antkiewicz-Michaluk et al. 2003). Additionally, intracerebral-administered rotenone (2 μg into the medial forebrain bundle, MFB) produced a considerable decrease in dopamine and dopamine metabolites content in the striatum and substantia nigra, without affecting the serotonin

system (Antkiewicz-Michaluk et al. 2004). Those changes were observed 21 days after the intracerebral injection of rotenone, which suggest a durable neurotoxic effect. Peripheral administration of 1MeTIQ (50 mg/kg i.p.) before and then daily for 21 days significantly reduced the fall of striatal dopamine concentration (Antkiewicz-Michaluk et al. 2004).

The above data suggest that 1MeTIQ is able to counteract the damaging action of dopaminergic neurotoxin, rotenone, and seems to be a potential neuroprotective agent.

3.6 Neuroprotection of 1MeTIQ Against Glutamate-Evoked Neurotoxicity

Recently, it was demonstrated that 1MeTIQ shares many activities with TIQ, and found that the compounds similarly inhibit free-radical generation in an abiotic system, as well as indices of neurotoxicity (caspase-3 activity and lactate dehydrogenase release) induced by glutamate in mouse embryonic primary cell cultures (Antkiewicz-Michaluk et al. 2006). However, in granular cell cultures obtained from 7-day-old rats, 1MeTIQ prevented the glutamate-induced cell death and $^{45}Ca^{2+}$ influx, whereas TIQ did not. Such profile of action of 1MeTIQ suggested specific effects of this compound on an excitatory amino acids (EAA) receptors. Additionally, it was shown in an in vivo microdialysis experiment that 1MeTIQ prevents kainate-induced release of excitatory amino acids from the rat frontal cortex (Antkiewicz-Michaluk et al. 2006).

Comparing the chemical structure of 1MeTIQ with other known compounds containing tetrahydroisoquinoline skeleton and their molecular mechanism of action, one can find similarities between 1MeTIQ and N-cetyl-1-(4-chlorophenyl)-6,7-dimethoxy-TIQ; and 1,1-pentamethylene-TIQ the derivatives which are non-competitive AMPA/kainate receptor antagonists and protect the animals in the maximal electroshock seizure, pentylenetetrazole, and audiogenic DBA/2 mice seizure models (Ferreri et al. 2004; Gitto et al. 2003). In fact, 1MeTIQ exerts the anticonvulsant effects increasing the threshold for electroconvulsions and potentiation, the antiseizure action of carbamazepine and valproate against maximal electroshock (Luszczki et al. 2006).

3.7 Conclusion

In the light of all these experiments 1MeTIQ offers a unique and complex mechanism of neuroprotection in which inhibitory effect on MAO connected with free radicals scavenging properties, and antagonism to the glutaminianergic system may play a very important role.

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Chapter 4
1-Methyl-1,2,3,4-Tetrahydroisoquinoline and Addiction: Experimental Studies

Lucyna Antkiewicz-Michaluk and Jerzy Michaluk

Contents

Abstract Drug abuse disorder is induced by a variety of substances and results from their interaction with the brain reward system. It is characterized by a high frequency of relapse, usually associated with craving. In this Chapter it is demonstrated that 1-methyl-1,2,3,4-tetrahydroisoquinoline, an endogenous compound with antidopaminergic and neuroprotective activity prevented the development of morphine dependence and morphine-induced abstinent syndrome as well as cocaine-induced reinstatement in cocaine-dependent, self-administering rats. The changes in catecholamine metabolism persist for a considerable period after cessation of

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cocaine self-administration suggesting a long-lasting functional impairment of dopamine and noradrenaline systems. In contrast, the changes in the serotonergic system are transient showing the lack of involvement of serotonin in long-term consequences of exposure to cocaine. The depression of dopaminergic activity in the limbic structures may be responsible for craving. The fact that 1MeTIQ elevates the concentration of dopamine preferentially in the limbic structures (nucleus accumbens) in cocaine-dependent rats may be responsible for its inhibition of reinstatement. The results strongly support the view that 1-methyl-1,2,3,4-tetrahydroisoquinoline has considerable potential as a drug for combating substance abuse disease through the attenuation of craving, and suggested a possibility of clinical application of 1MeTIQ at least in morphine and cocaine addiction.

Keywords 1-Methyl-1,2,3,4-tetrahydroisoquinoline • Addiction • Animal models • Cocaine • Self-administration • Morphine dependence • Rat brain • Monoamine metabolism • MAO inhibition

Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
COMT	Catechol-O-methyltransferase
DA	Dopamine
DOPAC	3,4-Dihydroxyphenylacetic acid
5-HT	Serotonin
5-HIAA	5-Hydroxyindoleacetic acid
HVA	Homovanillic acid
MAO	Monoamine oxidase
1MeTIQ	1-Methyl-1,2,3,4-tetrahydroisoquinoline
MHPG	3-Methoxy-4-hydroxyphenylglycol
3-MT	3-Methoxytyramine
NA	Noradrenaline

4.1 Addiction: A General View

Addiction is a complex disease process of the brain which results from recurring drug intoxication and is modulated by genetic, developmental, experiential, and environmental factors. Drug addiction is one of the most difficult medical and social problems in industrialized countries; furthermore no effective pharmacotherapy has been available so far. Until recently, it was believed that addiction was associated with neuroplasticity in the corticostriatal brain circuitry, which is important for adaptive behavior and predominantly involved reward processes mediated by limbic circuits, whereas results from recent neuroimaging studies have implicated additional

Table 4.1 The pharmacological actions and reinforcing properties of different drugs of abuse

Drugs	Actions	
Cocaine	Inhibitor of monoamine reuptake transporters	Reinforcing activity by an increase of the extraneuronal concentration of DA, NA, 5-HT (Koob and Nestler 1997; Edwards and Koob 2010; Kuhar et al. 1991)
Opiates	Agonist at μ, δ, and κ receptors	Activation of μ and δ receptors mediate the reinforcing action of opiates (Mansour et al. 1995)
Amphetamine	Stimulates DA and NA release	Reinforcing activity (Elverfors and Nissbrandt 1992)
Nicotine	Agonist at nicotinic acetylcholine receptor	Reinforcing activity (Biała and Budzynska 2008; Risinger and Roger 1995; Biała 2003)
Ethanol	Facilitates $GABA_A$ receptor function and inhibits NMDA receptor	Reinforcing activity (Biała and Budzyńska 2010; Hoshaw and Lewis 2001)
Hallucinogens	Partial agonist 5-HT_{2A} receptors	Reinforcing activity (Fantegrossi 2007)
Phencyclidine (PCP)	NMDA receptor antagonist	Reinforcing properties (Rodefer and Carroll 1999)

brain areas, especially the frontal cortex (Goldstein and Volkow 2002). The World Heath Report 2002 stated that 8.9% of the total burden of the disease was caused by the use of psychoactive substances (WHO, Neuroscience of psychoactive substance use and dependence, World Health Organization, Geneva (2004). Drug addiction is often defined by the pharmacological terms: *tolerance, sensitization, dependence, and withdrawal. Tolerance* refers to the phenomenon where repeated administration of a drug at the same dose causes a diminishing effect or a need for an increasing drug dose to produce the same effect. *Sensitization* refers to the opposite condition, where repeated administration of the same drug dose produces an escalating effect. Interestingly, the same drug can simultaneously evoke tolerance and sensitization to its numerous diverse effects (e.g., in the case of morphine, tolerance to its analgesic effect and sensitization to its locomotor effect). *Dependence* is defined as a need for continual drug exposure to avoid a *withdrawal* syndrome, which is characterized by physical or motivational disturbances when the drug is withdrawn.

The neurobiological changes that accompany drug addiction have not been understood so far; however, drugs of abuse are unique in terms of their reinforcing properties (Table 4.1). Dopaminergic mechanisms are a traditional target in the field of addiction, since the acute rewarding effects of addictive drugs are mediated by enhancing dopamine transmission; moreover, dopamine release reinforces reward learning (Berridge and Robinson 1998; Kelley 2004a). Both natural rewards and drugs of abuse appear to use the same systems within the brain to influence and reinforce behavior; furthermore, it is well known that these systems are involved in learning and memory, particularly in connecting motivations and memories with behaviors. A question arises about the neurobiological substrate of reward. The nucleus accumbens (NAc) as a ventral striatum is considered to be crucial point of

integration of information by receiving emotional and cognitive inputs, and by projecting to motor output regions (Mogenson et al. 1980; Kelley 2004b). The NAc, along with the hippocampus, frontal cortex (FCx), and basolateral amygdala receives dopamine input from the ventral tegmental area (VTA); furthermore, as it has been shown by many authors, the majority of dopamine neurons that innervate the forebrain are located in the midbrain, specifically in the VTA and substantia nigra (SN) (Fallon and Loughlin 1995; Pitkanen 2000). The SN innervates the dorsal striatum (caudate-putamen), whereas the VTA provides an input to the rest of the forebrain, including the ventral striatum (NAc), FCx, amygdala, and hippocampus. Early theories on drugs of abuse and natural rewards suggested that activation of dopamine neurons in VTA, and the release of dopamine in target structures signaled reward, especially in the NAc (Di Chiara 2002; Ungless 2004). However, aversive stimuli also increase dopamine release in a variety of brain structures, which indicates a role of dopamine beyond reward (Inglis and Moghaddam 1999). However, it is noteworthy that some evidence points to differential of dopamine responses to aversive vs. rewarding stimuli (Schultz 2002). Special interest is the fact that common effects produced by different drugs of abuse with a diverse pharmacological action including alcohol, cocaine and heroin, lead to an increase in the release of dopamine in the NAc which is critical for drugs to reinforce behavior (Table 4.1). Some recent studies have also shown that glutamate system and its release is an important factor in drug addiction, and that imbalance in glutamate homeostasis engenders changes in neuroplasticity, which impair communication between the prefrontal cortex and the NAc (Kalivas 1995; Ma et al. 2006; Nagy 2004; Popik et al. 1998).

4.1.1 Animal Models of Drug Addiction

As the basic mechanisms underlying addiction are similar in animals and humans, the animal models may be used for studying the mechanisms of substance abuse disease (Markou et al. 1993; Ranaldi and Roberts 1996). One major model is drug-induced *locomotor sensitization*. In rodents, acute administration of many drugs of abuse increase the animal locomotor activity, and after chronic treatment produce even bigger increases in locomotor activity. Sensitization is a phenomenon characterized by enhancement of behavioral responses (locomotor hyperactivity, stereotypy, positive reinforcing effects) to the readministered psychostimulant (challenge dose) after discontinuation of its repeated treatment (Kalivas et al. 1988; Pierce and Kalivas 1997; Robinson and Berridge 1993). The above described phenomenon seems interesting, since it may be caused by the same mechanisms that are responsible for psychoses or craving for drugs of abuse in humans addicted to cocaine or other psychostimulants (Robinson and Berridge 1993).

Another, frequently used model is conditioned place preference (CPP). Animals are administered a drug of abuse before being placed in one chamber of a two-chamber apparatus. Then, they are given a control injection before being put into the

other chamber. By repeating this procedure, the animals learn to associate one chamber with the drug of abuse. They are tested by being allowed to freely explore the entire apparatus; the animals that have learned the drug-chamber association spend more time in the drug-associated chamber. This model is particularly useful for investigating contextual learning which take place during drug consumption (Hoffman 1989; Popik and Wróbel 2002).

The best animal model of addiction is *self-administration*. In this paradigm, animals are trained to perform a response (usually, pressing a lever) in order to receive a small amount of the drug. In contrast to sensitization and CPP, when experimenters administer a drug of abuse, the advantage of the latter model is like in the case of a human who must perform certain actions to obtain and consume a drug of abuse – that an animal has to exhibit a particular behavior in order to receive the drug. Self-administration is called a "gold-standard" of addiction.

Drugs of abuse, especially their repeated administration, alter the functioning of a number of structures in the brain, of which the NAc and FCx are particularly important due to their apparent role in addiction.

4.2 Cocaine Addiction

Cocaine, a potent inhibitor of monoamine transporters (Woolverton and Johnson 1992), belongs to the most powerful addictive substances and its abuse poses a high risk of relapse (Carroll et al. 1994). It has been reported that even a single injection produces persistent neurochemical changes (Guan et al. 1995); moreover, a long-lasting changes changes have also been observed after withdrawal from the chronic administration of cocaine (Kuhar and Pilotte 1996). The factors or molecular processes that are responsible for relapses of cocaine abuse have not been fully understood so far. An overwhelming desire to take drugs (craving) is regarded as a main cause of relapse (Koob and LeMoal 2001).

In addiction studies, one of the critical issues is the use of an appropriate animal model for the behavior under study. The animal model that seems to be the most adequate for studying the craving and relapse phenomena is an extinction/reinstatement task in self-administration procedures. In this paradigm, the animals learn to self-administer a solution of a drug (e.g., cocaine) by pressing "the active lever" installed in the experimental cage. Cocaine self-administration serves as a *reward*, but the response may be strengthened by pairing it with the conditioned stimulus (e.g., light + tone). When after establishing a stable response rate the drug solution is replaced with saline, the responding rate decreases and the animal enters the *extinction phase*. Administration of a priming dose of cocaine during this phase results in *reinstatement*, expressed as the resumption of pressing the active lever. Furthermore, the presentation of the conditioned stimulus alone is sufficient to cause reinstatement of drug-seeking behavior measures as an increase in the number of lever presses in the absence of cocaine; however, such an effect is weaker than in the case of drug reinstatement (Cervo et al. 2003; Antkiewicz-Michaluk et al. 2006a, 2007).

In rats self-administering cocaine, the rate of monoamines (dopamine, noradrenaline, and serotonin) metabolism as well as their turnover was depressed (Antkiewicz-Michaluk et al. 2006a, b, 2007). Activation of a receptor (e.g., dopamine, noradrenaline, serotonin) by cocaine which is a potent inhibitor of monoamine transporters during the stabilized self-administration (when cocaine is present in the brain) would result in a feedback inhibition of neurotransmitter release (owing to activation of autoreceptors) and, consequently, the depression of neurotransmitter metabolism without significant changes in neurotransmitter concentration in the neuron (Antkiewicz-Michaluk et al. 2006a, b; Karoum et al. 1990; Trulson and Ulissey 1987). In the extinction period catecholaminergic neurons show signs of long-lasting impairment, while the extracellular concentration of dopamine is reduced in the limbic areas of the central nervous system (Kuhar and Pilotte 1996; Cervo et al. 2003; Antkiewicz-Michaluk et al. 2006a, b). Interestingly, the stimulus associated with cocaine availability (e.g., light + tone) activates the catecholaminergic system after the extinction procedure and causes reinstatement of self-administration.

4.3 1-Methyl-1,2,3,4-Tetrahydroisoquinoline (1MeTIQ) and Cocaine Addiction

4.3.1 1MeTIQ: A Partial Dopamine Agonist and Neuroprotectant in the Mammalian Brain

1,2,3,4-Tetrahydroisoquinoline (TIQ) and its close methyl-derivative, 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ) are both present in the food and mammalian brain which are believed to play a physiological role as natural regulators of the dopaminergic neurotransmitter system (Antkiewicz-Michaluk et al. 2000, 2001). One of the most evident physiological roles proposed is antidopaminergic activity, which may control the effect of temporary, excessive dopaminergic stimulation (Antkiewicz-Michaluk and Vetulani 2001; Vetulani et al. 2003a, b). It was initially believed that tetrahydroisoquinolines, structurally resemble 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), were neurotoxic; however, it was later discovered that some of them exerted a neuroprotective effect. The action of different compounds of this group ranges from neurotoxicity to neuroprotection, as exemplified by the neurotoxin 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1BnTIQ), and the neuroprotectants, TIQ and its close methyl derivative, 1MeTIQ (Abe et al. 2005; Antkiewicz-Michaluk et al. 2006a, b; McNaught et al. 2001; Nagatsu 1997; Wąsik et al. 2009). Importantly, various TIQs differ in their effect on dopamine catabolic pathways: the monoamine oxidase (MAO)-dependent and the catechol-O-methyltransferase (COMT)-dependent one. The neurotoxic, 1BnTIQ shifts dopamine catabolism toward oxidative desamination, whereas neuroprotectants studied (TIQ and 1MeTIQ) strongly inhibit the MAO-dependent pathway and shift the

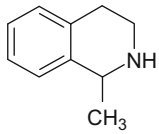

Fig. 4.1 Chemical structure of 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ)

COMT-dependent catabolism toward O-methylation (Antkiewicz-Michaluk et al. 2001). In fact, in vitro studies have shown that both compounds are inhibitors of monoamine oxidase A and B in rat brain structures (Patsenka and Antkiewicz-Michaluk 2004). Since oxidative desamination generates free hydroxyl radicals, differences in the biochemical effects of tetrahydroisoquinolines could account for their diverse neurotoxic/neuroprotective profiles of action (Patsenka et al. 2004). The most evident neuroprotection in CNS was described for 1MeTIQ (Tasaki et al. 1991; Antkiewicz-Michaluk et al. 2003, 2004, 2006a, b; see Chap. 3), a compound which has an interesting pharmacological profile and may play a role of a natural agent protecting the brain against Parkinson's disease (Fig. 4.1).

1MeTIQ which is present in the brain, is a mixture of (R)- and (S)-enantiomers enzymatically synthesized from 2-phenylethylamine (PEA) and pyruvate by the 1MeTIQ-synthesizing enzyme, a membrane-bound protein localized in the mitochondrial synaptosomal fraction (Yamakawa and Ohta 1997, 1999; Yamakawa et al. 1999). 1MeTIQ was shown to act as an antidopaminergic agent, but in contrast to typical neuroleptics, it did not induce catalepsy in animals (Antkiewicz-Michaluk et al. 2000). In functional studies 1MeTIQ inhibited the apomorphine-induced hyperactivity at doses at which it had no effect on spontaneous locomotor activity of rats (Antkiewicz-Michaluk et al. 2001). In addition, 1MeTIQ also displaced agonists, but not antagonists, of the dopamine receptor from their binding sites with an affinity comparable to that of dopamine (Antkiewicz-Michaluk et al. 2007). This observation suggests that the compound shows affinity for the agonistic (active), but not antagonistic, conformation of dopamine receptors. Summing up, 1MeTIQ exerts effects characteristic of dopamine partial agonists. Regardless of the mechanism of action of drugs of abuse, the essential role of the mesolimbic dopaminergic system in addiction has been well established (Goldstein and Volkow 2002; Grimm et al. 2003; Moore et al. 1998a, b); to this end, several antidopaminergic drugs were tested as potential antiabuse agents (Berger et al. 1996; Smelson et al. 2004).

Although, neuroleptics were previously found not to be useful in this respect, partial agonists of the dopamine D_2 and D_3 receptor aroused some hopes (Campiani et al. 2003; Le Foll et al. 2005; Mach et al. 2004). Furthermore, a dopamine reuptake inhibitor could be expected to partially substitute for cocaine and other drugs of abuse, hence self-administration would be diminished and craving minimized (Ritz et al. 1987; Wilcox et al. 2000; Carroll 2003; Carroll et al. 2004). This type of substitution pharmacotherapy has been found to be highly effective in the treatment of nicotine and heroin addiction (methadone). Hence, studies of partial agonists with an antidopaminergic profile of action different from that of classic neuroleptics seem justified. In the light of the above data, 1MeTIQ is an interesting candidate for future clinical studies.

A vast body of evidence indicates that, apart from the dopaminergic system also glutamatergic system is involved in the addiction to drugs of abuse (see Table 4.1). Hence of special interest are the observations that 1MeTIQ antagonizes the kainate-induced release of glutamate and aspartate in rat frontal cortex and shows neuroprotection against the excitotoxicity produced by glutamate in culture cells (Antkiewicz-Michaluk et al. 2006a, b). Additionally, 1MeTIQ antagonizes the MK-801-produced behavioral and neurochemical effects (Pietraszek et al. 2009) and shares tolerance to excitotoxicity in rat with some well-established uncompetitive NMDA receptor antagonists (Kuszczyk et al. 2010). The latest results reveal a new mechanism of the 1MeTIQ-evoked neuroprotection based on the induction of neuronal tolerance to excitotoxicity.

4.3.2 Effect of 1MeTIQ on the Cocaine-Induced Locomotor Sensitization, Self-Administration, and the Expression of Cocaine Reinstatement

In rodents, acute administration of the drug of abuse: cocaine, amphetamine (psychostimulants), and opiates increases the locomotor activity of animals. Repeated administration of the drug of abuse induces neurobiological changes, such that later (e.g., after 10 days of withdrawal) acute administration of the drug produces even greater increase in locomotor activity, called sensitization. Both behavioral sensitization, self-administration, and drug-reinstatement of seeking behavior are the major models of drug addiction (Pierce and Kalivas 1997). The compounds which antagonize locomotor sensitization and self-administration in animals may demonstrate anti-addictive properties in a clinic (Narayanan et al. 1996).

The exogenously applied 1MeTIQ, an endogenous substance constantly present in the brain significantly antagonized the cocaine-induced locomotor sensitization, cocaine self-administration and cocaine-induced reinstatement of seeking behavior (Filip et al. 2007; Wąsik et al. 2010). The phenomenon is of interest, since it might be caused by the same mechanisms as those responsible for psychoses or craving for drugs of abuse in humans abusing cocaine or other psychostimulants (Robinson and Berridge 1993; Segal et al. 1981). Both clinical and preclinical studies indicate that the behavioral response to cocaine including the discriminative stimulus and rewarding effects as well as reinstatement of cocaine seeking behavior depend on the drug ability to block the dopamine transporter (Di Chiara 1995; Heidbreder and Hagan 2005). Since, 1MeTIQ produced parallel decreases in cocaine self-administration and cocaine-induced relapse, the compound may suppress the motivation for drug seeking by decreasing the reinforcing effects of cocaine, and generally by attenuation the reinforcing effect of drugs of abuse.

In fact, activation of both the dopaminergic and glutaminergic systems has significance in altering the maintenance of cocaine self-administration (Cornish et al. 1999; Pulvirenti et al. 1992), and drug-priming induced reinstatement of cocaine

seeking (Ito et al. 2002; Kalivas and McFarland 2003). 1MeTIQ's inhibitory mechanism on cocaine maintained responding and relapse may include complex interaction with both dopaminergic and/or glutaminergic transmission (as it was shown above, in part 3.1.).

4.3.3 Neurochemical Changes Produced by 1MeTIQ in Cocaine-Dependent Rats

Cocaine, is a potent inhibitor of monoamine transporters, belongs to the most powerful addictive substances in humans and its abuse has a high risk of relapse (Gawin 1991). The studies on the involvement of biogenic amines in cocaine addiction have shown a contribution of dopamine and serotonin to the maintenance of cocaine self-administration, extinction, and reinstatement of drug-seeking behavior. Dopamine was the most extensively investigated amine, possibly because of its assumed role as the main neurotransmitter of reward (Di Chiara et al. 2004). How it was demonstrated by several authors during cocaine self-administration, the extracellular concentrations of dopamine and serotonin in the striatum increase (Di Ciano et al. 1995; Gratton and Wise 1994; Wąsik et al. 2010), but the rate of monoamines metabolism and the concentrations of their metabolites in brain structures decrease (Antkiewicz-Michaluk et al. 2006a, b). The functional significance of the observed depression of the level of monoamine metabolites requires explanation. The determination of changes in metabolism rate yields information about efficiency of the neurotransmitter system. Depending on the state of receptor and the rate of synthesis of a neurotransmitter, the changes in metabolite levels in the same direction may have different consequences. Activation of a receptor (e.g., dopamine receptor) during stabilized self-administration of cocaine would result in a feedback inhibition of neurotransmitter release by activation of autoreceptor and, the depression of neurotransmitter metabolites without significant changes in neurotransmitter concentration in the neuron. This is reflected by a decrease in the neurotransmitter metabolism index. As the stimulation is indirect, through inhibition of neurotransmitter reuptake, the interaction between the neurotransmitter and receptor is enhanced rather than depressed. On the contrary, during cocaine withdrawal, dopamine and noradrenaline concentrations as well as concentration of their metabolites were diminished, suggesting cocaine-induced impairment in the function of catecholamine neurons, what leads to decrease in the synthesis and release of the neurotransmitters (Parsons et al. 1995; Weiss et al. 1992; Antkiewicz-Michaluk et al. 2006a, b).

Much less attention was focused on the involvement of noradrenaline in cocaine reward. It has been reported that multiple cocaine administration depresses the responsiveness of the alpha2-adrenoceptor, as measured by the decline in the growth hormone response to clonidine (Baumann et al. 2004). Moreover, Valentino and Curtis (1991) reported the inhibition of firing of locus coeruleus neurons after long-term cocaine treatment.

While catecholamines seem to be involved in cocaine addiction, the role of serotonin seems to be limited. Specific serotonin agonists do not seem to have significant reinforcing efficacy (Locke et al. 1996). The clinical findings also indicate the inefficiency of serotonergic manipulation in combating cocaine dependence (Lima et al. 2003). Although, serotonin metabolism is inhibited in the presence of cocaine, in contrast to alterations in catecholaminergic system, the change is transient.

Basically, a chronic cocaine self-administration similar to passive administration suppresses the metabolism – both synthesis and release of monoamines in several brain structures (Karoum et al. 1990; Trulson and Ulissey 1987; Antkiewicz-Michaluk et al. 2006a, b). The changes in catecholamine metabolism persist for a considerable period after cessation of cocaine self-administration suggesting a long-lasting functional impairment of dopamine and noradrenaline systems. In contrast, the changes in the serotonergic system are transient showing the lack of involvement of serotonin in long-term consequences of exposure to cocaine (Antkiewicz-Michaluk et al. 2006a, b).

4.3.4 Neurochemical Effect of 1MeTIQ During Cocaine Reinstatement

The suggestion that 1MeTIQ is a potential antiabuse agent is due to the fact that the drugs that reduce cocaine-seeking behavior also reduce cocaine craving (Fuchs et al. 1998; Baker et al. 2001). The possible antiabuse properties of 1MeTIQ are particularly interesting, as the compounds of this group are proposed to act as regulators of brain homeostasis (Antkiewicz-Michaluk et al. 2000; Vetulani 2001). The question arises whether 1MeTIQ can reach the brain in concentrations producing pharmacological effects. In contrast to catechol tetrahydroisoquinolines (e.g., salsolinol), noncatechol tetrahydroisoquinolines as TIQ and 1MeTIQ penetrate to the brain easily and induce a variety of central effects. The native concentrations of tetrahydroisoquinolines in the brain are in the nanomolar range, and the highest concentration was observed in substantia nigra: in the monkey substantia nigra 1MeTIQ reaches up to 500 pmol/g tissue (Yamakawa et al. 1999), and it is depressed in aged rats (Ayala et al. 1994). No direct measurements of 1MeTIQ concentration in the brain after parenteral administration of the compound have been carried out, but its close congener, TIQ after administration of 40 mg/kg to the rat reached the concentration of 250 nmole/g (Lorenc-Koci et al. 2004).

In cocaine-dependent rats, the intraperitoneal administration of a single reinstatement evoking cocaine dose causes the inhibition of dopamine metabolism in the brain structures of rat. This is possible because of the consequence of the dopamine transporter blockade, and the feedback response of the nerve ending to an increase in the concentration of dopamine in the vicinity of the receptor sites. It should be added that priming dose of cocaine also affects other monoaminergic neurons, and inhibits also the metabolism of noradrenaline and serotonin. As in the case of dopamine, a feedback mechanism seems to be involved (Antkiewicz-Michaluk et al. 2007).

In cocaine-dependent rats receiving a priming dose of cocaine in the presence of previously administered 1MeTIQ, the concentration of dopamine in the limbic structures was significantly higher than in the rats receiving cocaine alone. It might be assumed that the blockade of reinstatement by 1MeTIQ is related to this effect (Antkiewicz-Michaluk et al. 2007). There is the long established view that depression of dopaminergic activity in the limbic structures may be responsible for craving (Rossetti et al. 1992; Little et al. 1996; Wise et al. 1990). The fact that 1MeTIQ elevates the concentration of dopamine preferentially in the limbic structures (nucleus accumbens) in cocaine-dependent rats, and at the same time inhibits dopamine metabolism in structures containing cell bodies (substantia nigra, VTA), may be responsible for its inhibition of reinstatement (Antkiewicz-Michaluk et al. 2007).

Another neurochemical action of 1MeTIQ, possibly related to its anticraving effect, is activation of the noradrenergic system in the brain. This effect may be related to the antagonistic action of 1MeTIQ on alpha2-adrenoceptors. Such an activity was described previously for other, closely related tetrahydroisoquinolines (Michaluk et al. 2002; Vetulani et al. 2003a, b). The ability of 1MeTIQ to increase the level of the main metabolite of noradrenaline in CNS, 3-methoxy-4-hydroxyphenylglycol (MHPG), as well as its extraneuronal metabolite, normetanephrine (NM) reflects the antagonistic effect of 1MeTIQ on the alpha2-adrenoceptor (Antkiewicz-Michaluk, unpublished data). Inhibition of alpha2-adrenoceptors would result in an increase in noradrenaline release from the nerve endings, and consequently activating the noradrenergic system.

In the light of the recent experimental data it looks that serotonin plays a less important role in the phenomenon of cocaine reinstatement. It was shown, that cocaine depresses serotonin metabolism only transiently, and that the changes do not persist throughout the withdrawal period in contrast to dopamine and noradrenaline systems (Antkiewicz-Michaluk et al. 2005).

4.4 1MeTIQ and Morphine Addiction

4.4.1 The Influence of 1MeTIQ on the Morphine-Induced Analgesia, Tolerance, and Abstinence Syndrome

Morphine acts through activation of opioid μ-receptors and produces antinociceptive effect called analgesia. It is well known that activation of opioid μ-receptors is closely related with inhibition of calcium uptake and this process is responsible for opioid-induced analgesia (Kamikubo et al. 1983; Chapman and Way 1982). It was presented that 1MeTIQ administered alone have shown a slight antinociceptive effect in the "hot-place" test in rats but coadministered in morphine strongly potentiated its analgesia (Wąsik et al. 2007; Vetulani et al. 2003a, b). Moreover, 1MeTIQ applied before each morphine injection completely inhibited the development of morphine tolerance, as well as prevented the naloxone-induced precipitation of the abstinence

syndrome (the head-twiches and the body weight loss) in the morphine-dependent rats (Wąsik et al. 2007). The question arises what was the mechanism responsible for 1MeTIQ-induced potentiation of morphine-analgesia, prevention of morphine-produced tolerance and abstinence syndrome? Some authors postulated that the activity of monoamine oxidaze (MAO), the enzyme crucial for monoamine and special dopamine catabolism, and the production of free radicals play a very important role in opiate analgesia, tolerance, and dependence (Garzon et al. 1979; Grassing and He 2005). In fact, it was shown that deprenyl, an irreversible inhibitor of MAOB and other antioxidants, such as vitamin C produced an increase in morphine antinociception and could prevent the development of morphine tolerance and physical dependence in rodents (Sanchez-Blazquez et al. 2000; Khanna and Sharma 1983). 1MeTIQ, a neuroprotective substance inhibits MAO and possesses free radicals scavenging properties (Antkiewicz-Michaluk et al. 2006a, b) how it was mentioned above, and this mechanism would be responsible for its antinociception and antiaddictive effects. Additionally, 1MeTOQ is also effective in prevention of morphine-induced place preference and of alcohol intake (Antkiewicz-Michaluk et al. 2005).

Moreover, it was also shown by many authors that morphine did not trigger effectively the processes leading to the development of tolerance and dependence when administered during Ca^{2+} channel blockade. Blockade of the voltage-dependent L-type Ca^{2+} channels effectively facilitates the analgesic action of morphine and prevents the behavioral and neurochemical signs of naloxone-precipitated abstinence syndrome (Contreras et al. 1988; Del Pozo et al. 1987; Michaluk et al. 1998). Another way for calcium influx to the cell is NMDA glutamate receptors which may be also involved in the induction of morphine sensitization (Vanderschuren and Kalivas 2000). It should be taken into account that 1MeTIQ prevented glutamate-induced cell death and $^{45}Ca^{2+}$ influx in granular cell cultures (Antkiewicz-Michaluk et al. 2006a, b). Thus, 1MeTIQ besides the inhibitory influence on the activity of MAO and free radical scavenging properties possesses also a mild activity at NMDA receptors.

4.5 Conclusions

Drug abuse disorder is induced by a variety of substances and results from their interaction with the brain reward system. Exogenous administration of 1MeTIQ, an endogenous compound with antidopaminergic and neuroprotective activity, completely inhibits the expression of reinstatement of cocaine self-administration, morphine abstinent syndrome and prevents the development of morphine tolerance, simultaneously potentiating morphine-induced analgesia in the rat. To explain the mechanism of the antiaddictive effects of 1MeTIQ, its wide spectrum of action in the CNS should be considered. Functional studies have shown that 1MeTIQ acts as an antidopaminergic agent but, in contrast to typical neuroleptics, it induces no sedation or catalepsy in animals. Moreover, 1MeTIQ indicates a direct interaction with the agonistic conformation of dopamine receptors, proposed for dopamine partial agonists. On the other hand, it also displays a moderate effect on the NMDA

receptor and the glutaminergic system, and offers neuroprotection against the glutamate-induced excitotoxicity in rat. This ability of 1MeTIQ may be of clinical importance and raises hope for its application in the neurodegeneration disease (e.g., Parkinson's disease) and addiction evoked by drugs of abuse.

In summary, the presented results strongly support the view that 1MeTIQ is a drug which has a considerable potential suitable to combat drug addiction, particularly through attenuation of the abstinence syndrome and craving.

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Part II
β-Carbolines as Neurotoxins and Neuroprotectants

Chapter 5
β-Carbolines as Neurotoxins

Tomás Herraiz

Contents

Abstract Tetrahydro-β-carbolines (THβCs) and β-carbolines (βCs) are bioactive naturally occurring indole alkaloids and structural analogs of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenylpyridinium (MPP^+) Parkinsonian neurotoxins. Humans are daily exposed to these compounds through the diet, smoking, and plants, and they are also found endogenously in human tissues and brain. βCs can be converted by *N*-methyltransferases occurring in the mammalian brain into *N*-methyl-β-carbolinium cations (βC^+s) which are neurotoxins. These βC cations have been detected in the human brain and share with MPP^+ several toxicological features such as inhibition of mitochondrial complex I, increase of ROS production and induction of cell apoptosis. As a result, they produce neurotoxicity in vitro and in vivo. Among βC cations, the most potent neurotoxins are

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N,*N*-dimethylated βC cations (2,9-diMe-βC$^+$s) that are produced from sequential *N*-methylation of βCs at the *N-2* (pyrido) and *N-9* (indole) nitrogens. Toxicity of 2,9-diMe-βC$^+$s approaches that of MPP$^+$ although it is less selective for dopaminergic cells. THβCs and βCs are metabolized by several cytochrome P450 enzymes to hydroxylated derivatives in a detoxification process that may compete with their bioactivation to neurotoxins by *N*-methyltransferases. Alternatively, *N*-methylTHβCs could be also bioactivated to aromatic βCs by heme peroxidases. These metabolic features affect the toxicological outcome of these compounds. Taken together, THβCs and βCs could be potential environmental and endogenous proneurotoxins that after bioactivation might play a role in the pathogenesis of neurodegenerative diseases in susceptible individuals. In the future, new studies are needed to clarify if the physiological levels of THβCs and βCs that reach and accumulate in the human brain may induce significant neurotoxicity in the sort and/or long-term or in contrast they may exert alternatively other bioactive actions including neuroprotection.

Keywords Tetrahydro-β-carbolines • β-Carbolines • MPTP • MPP$^+$ • Neurotoxins • Dopaminergic cell culture • MAO • N-methyltransferase • Cytochrome P450 • Free radicals • Mitochondrial function • Cell culture

5.1 Introduction

β-Carboline alkaloids (βCs) (9*H*-pyrido-(3,4-*b*)indole) are naturally occurring indole compounds that exhibit a broad range of biological and pharmacological activities including antimicrobial, antiviral, antioxidant, and antitumoral actions. They also exert a variety of psychoactive, physiological and neurological effects such as alteration of brain neurotransmitters, changes in body temperature, convulsion, vascular relaxation, antidepressant actions, and effects on drug withdrawal and appetite (Airaksinen and Kari 1981a, ; Rommelspacher et al. 1991a; Herraiz 2008; Robinson et al. 2003). Simple molecules of βCs have been detected endogenously in mammalian tissues and brain (i.e., called "mammalian" alkaloids) and its presence related with pathophysiological conditions (Airaksinen and Kari 1981b; Rommelspacher et al. 1991b; Parker et al. 2004; Buckholtz 1980). Mammalian βCs might function as neuromodulators via effects on monoamine oxidase (MAO), monoamine uptake and interaction with brain serotonin, benzodiazepine, dopamine, and opiate receptors and imidazoline-binding sites (Airaksinen and Kari 1981a; Rommelspacher et al. 1991a, 1994; Robinson et al. 2003; Buckholtz 1980; Herraiz and Chaparro 2005). Concerning toxicology, βCs have received attention as co-mutagens, precursors of mutagens and as neurotoxicants (Herraiz 2008; Totsuka et al. 1998; Hamann et al. 2006; Collins and Neafsey 1985). Additionally, β-carboline alkaloids occur in foods, tobacco smoke and medicinal plants such as *Peganum harmala* and *Banisteriopsis caapi* (Herraiz 2004a, b, 2008; Herraiz et al. 2010), suggesting that environmental sources (i.e., diet and smoking) contribute to the presence of these alkaloids in the human tissues and fluids. Human exposure to βCs is a current matter of interest because these compounds exert

many biological and toxicological actions and may accumulate in the brain. The current chapter provides an overview on βC alkaloids as putative neurotoxins involved in neurodegenerative diseases.

5.2 MPTP Neurotoxin and Its β-Carboline Analogs

In the late 1970s, idiopathic Parkinson's disease (PD) was reported in drug abusers after self-administration of a meperidine analog contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al. 1983) (Fig. 5.1). MPTP destroys nigrostriatal dopaminergic cells in the substantia nigra (dopamine depletion) causing irreversible Parkinsonism in humans, non-human primates, and rodents. This neurotoxin readily crosses the blood–brain barrier and is bioactivated by astrocytes and glial cells to pyridinium species which are directly acting neurotoxins in dopaminergic cells. Bioactivation of MPTP is carried out by the MAO-B that catalyzes its conversion to 1-methyl-2,3-dihydro-4-phenylpyridinium ($MPDP^+$) (Heikkila et al. 1984; Herraiz et al. 2006). $MPDP^+$ undergoes two electron oxidation by autooxidation, disproportionation, or oxidation catalyzed by enzymes to give the directly acting neurotoxicant 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is selectively uptaken into the nigrostriatal dopaminergic neurons via the high-affinity dopamine transporter (DAT). Within dopaminergic cells, MPP^+ concentrates in the

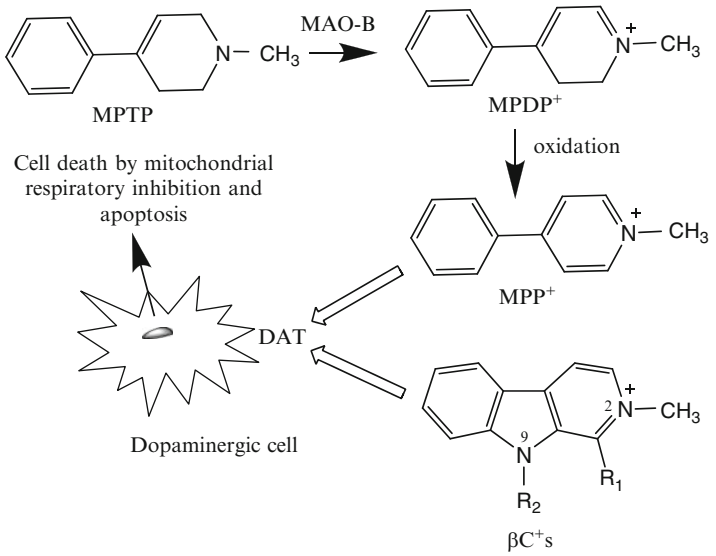

Fig. 5.1 Bioactivation process of MPTP neurotoxin to $MPDP^+$ by MAO-B in the brain. $MPDP^+$ is oxidized to MPP^+ which is a direct-acting neurotoxicant producing dopaminergic cell death through mitochondrial respiratory inhibition, ROS generation, and apoptosis. β-Carbolinium cations (βC^+s) are neurotoxic analogs of MPP^+. R_1 and R_2 are H or CH_3

mitochondrial matrix inhibiting electron transport chain at the complex I level and leading to the cessation of oxidative phosphorylation, ATP depletion, increased production of reactive oxygen species (ROS), mitochondrial dysfunction, and neuronal cell death by apoptosis (Przedborski and Vila 2003). Bioactivation by MAO-B is essential to generate Parkinsonism in animals exposed to MPTP and neurotoxicity is blocked by MAO-B inhibitors such as deprenyl (Heikkila et al. 1984), underlying the utility of inhibitors of this enzyme as potential neuroprotective agents (Herraiz et al. 2009).

The discovery of MPTP stimulated the hypothesis that PD and perhaps other neurodegenerative diseases are initiated in genetically predisposed subjects by unknown environmental or endogenous toxins. PD is the second most frequent neurodegenerative disorder and its predominant neuropathological feature is the degeneration of dopaminergic neurons in the substantia nigra. Epidemiological studies suggest that PD is associated with exposure to environmental factors such as rural life, pesticides, and industrial solvents (Di Monte et al. 2002). Since the discovery of MPTP, other chemical toxins such as rotenone, 6-hydroxydopamine and pesticides (paraquat, diquat) have been used to generate experimental Parkinsonism. Simultaneously, in the 1980s new research appeared suggesting that naturally occurring β-carbolines could be MPTP-like neurotoxins involved in PD (Collins and Neafsey 1985) (Fig. 5.1). Tetrahydro-β-carbolines (THβCs) show structural resemblance to MPTP (including a nitrogen bridge) whereas aromatic βC cations (β-carbolinium) show structural similarity to MPP^+ (Fig. 5.2). Interestingly, MPP^+ and βC cations share several functional and toxicological properties. Both are substrates for DAT and inhibit dopamine uptake (Storch et al. 2004b; Drucker et al. 1990), inhibit mitochondrial respiration at complex I level (Albores et al. 1990; Collins et al. 1992), increase ROS, are toxic to neuron cell cultures inducing apoptosis (Hamann et al. 2006), and produce neurotoxicity in mice and rats generating bradykinesia, reducing dopamine content in the striatum and midbrain and diminishing tyrosine hydroxylase immunoreactive cells (Matsubara et al. 1998a; Neafsey et al. 1989; Collins et al. 1996). In contrast to MPTP/MPP^+, βCs do occur in nature and have been detected in the human brain. Therefore, although $βC^+$s are usually less toxic than MPTP/MPP^+, they might still contribute to the degeneration of dopaminergic neurons during chronic exposure in predisposed subjects (Wernicke et al. 2007; Matsubara et al. 1992b). Recent results indicate that quaternary $βC^+$s containing both 2*N*- and 9[indole]-*N*-methyl groups (e.g., 2,9-diMe-$βC^+$s) (Collins et al. 1992; Collins et al. 1996) are the most potent carboline-based neurotoxic entities (Hamann et al. 2006; Collins et al. 1992; Collins et al. 1996; Wernicke et al. 2007). 2,9-diMe-$βC^+$s are potent mitochondrial inhibitors with neurotoxic effects resembling MPP^+, although with lower selectivity to dopaminergic cells (Fig. 5.1). β-Carbolinium cations ($βC^+$s) would be produced by *N*-methylation from hydrophobic and blood–brain barrier-permeable βCs and/or THβCs. A number of factors may affect the neurotoxicity of βCs, including their toxic potency but also their bioactivation, metabolism, DAT transport, and the occurrence of exogenous and endogenous βCs. They are reviewed below.

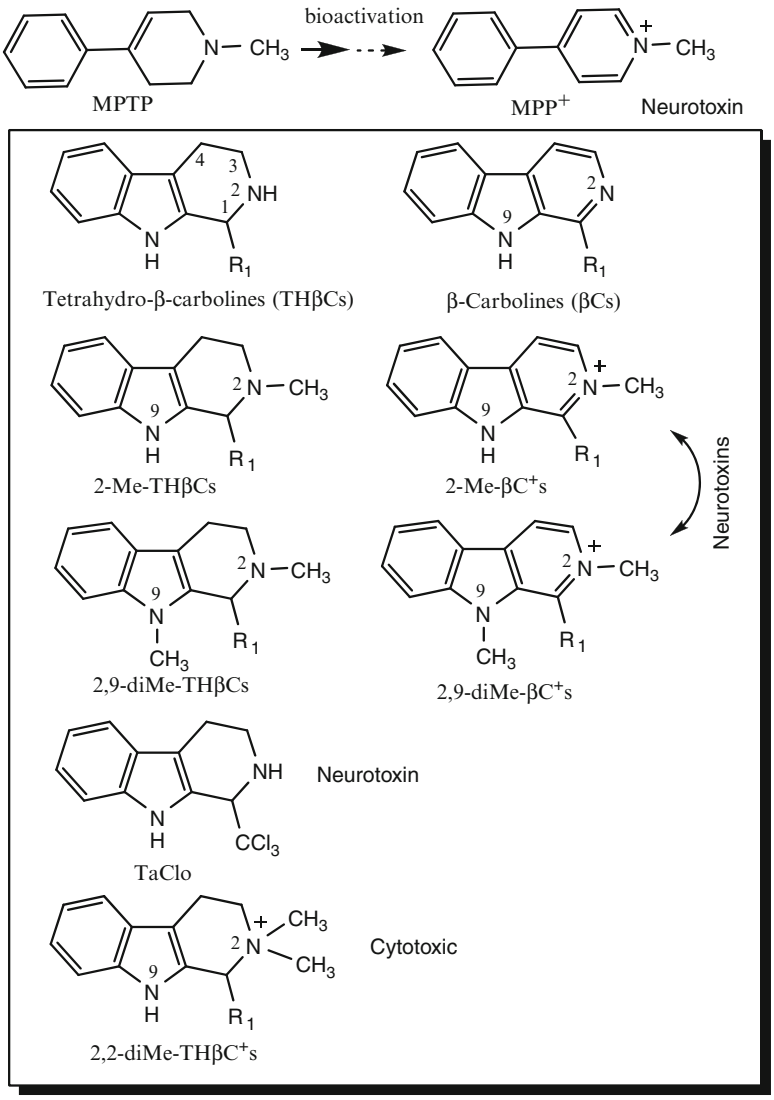

Fig. 5.2 THβCs and βCs are structural analogs with an indole nitrogen bridge of MPTP and MPP^+ neurotoxins. 2-Me-βC^+s and 2,9-diMe-βC^+s are functional neurotoxin analogs of MPP^+. $R_1 = H$, norharman series for βCs and tetrahydronorharman series for THβCs; $R_1 = CH_3$, harman series for βCs and tetrahydroharman series for THβCs. TaClo neurotoxin is a halogenated THβC. 2,2-diMe-THβC$^+$s cations are cytotoxic THβCs (Wernicke et al. 2007)

5.3 Bioactivation and Oxidative Metabolism of β-Carbolines

5.3.1 N-Methylation of β-Carbolines

In order to become neurotoxins, neutral and hydrophobic βCs are converted (i.e., bioactivated) into *N*-methylated βCs analogs of MPP^+ which are sequestered within the brain due to its cationic nature. βCs are bioactivated into neurotoxins by *N*-methyltransferases (NMTs) and a number of NMT enzymes catalyzing the *N*-methylation of βCs in both the *N*[2]- and *N*[9]-nitrogens to give β-carbolinium cations (βC^+s) have been already reported (Collins and Neafsey 1998; Gearhart et al. 1997). NMT activities have been detected in human brain (Collins and Neafsey 1998; Matsubara et al. 1993; Gearhart et al. 2000, 2002a) as well as in bovine, guinea pig, and rat brain (Matsubara et al. 1992a, b; Gearhart et al. 1997; Collins et al. 1992). The reaction requires *S*-adenosyl-L-methionine (SAM) as a methyl donor and the bioactivation process involves the sequential *N*-methylation of βCs, first in the 2β- and then in the 9[indole] nitrogen to give the highly neurotoxicant 2,9-dimethylated βC cations (2,9-diMe-βC^+) (Fig. 5.3). The 2-*N*-methylation activity would have an initial key role in trapping hydrophobic pyridoindoles within the brain (Collins and Neafsey 1998). Following methylation at *N*-2, the βC is partly in the form of anhydrobase which facilitates the subsequent *N*-methylation at the indole nitrogen to give 2,9-dimethylated βC cations. Although *N*-methylation may occur in both nitrogens of βC, methylation in the 9[indole] nitrogen appears to be the rate-limiting step in the development of toxicity (Collins et al. 1992; Matsubara et al. 1998a). Alternatively, a bioactivation process by NMT may also occur in THβCs (Fig. 5.3). A 2-*N*-methylation activity at the tetrahydropyrido ring to form 2β-*N*-methylated THβCs was detected in guinea pig and rat brain (Collins and Neafsey 1998; Matsubara et al. 1992a). However, in contrast to aromatic βCs, *N*-methylation at the 9-nitrogen has not been observed so far for THβCs (Matsubara et al. 1992a). Nevertheless, alternative routes of bioactivation might occur for THβCs such as an oxidation to aromatic βCs (Herraiz et al. 2007).

Sequential *N*-methylation of endogenous βCs might be an unique route to give toxic *N*-2,*N*-9-dimethylated β-carbolinium ions in vivo and the presence of these compounds in the human brain has been hypothesized as a causative factor in the pathogenesis of PD (Matsubara et al. 1993, 1998a). Higher levels of *N*-methylated βC cations were found in cerebrospinal fluid (CSF) of Parkinsonian patients than in age-matched controls (Matsubara et al. 1995). Hypothetically, an excess or aberrant *N*-methylation of βCs might activate neutral βCs into neurotoxins playing a role in the pathogenesis of PD (Gearhart et al. 1997, 2000; Aoyama et al. 2000; Matsubara et al. 2002). NMT enzymes with βC *N*-methylating capacity were significantly higher in CSF of younger patients suffering PD compared with control (Matsubara et al. 2002) and the expression of a nicotinamide *N*-methyltransferase with ability to *N*-methylate βCs was elevated in idiopathic PD (Aoyama et al. 2000;

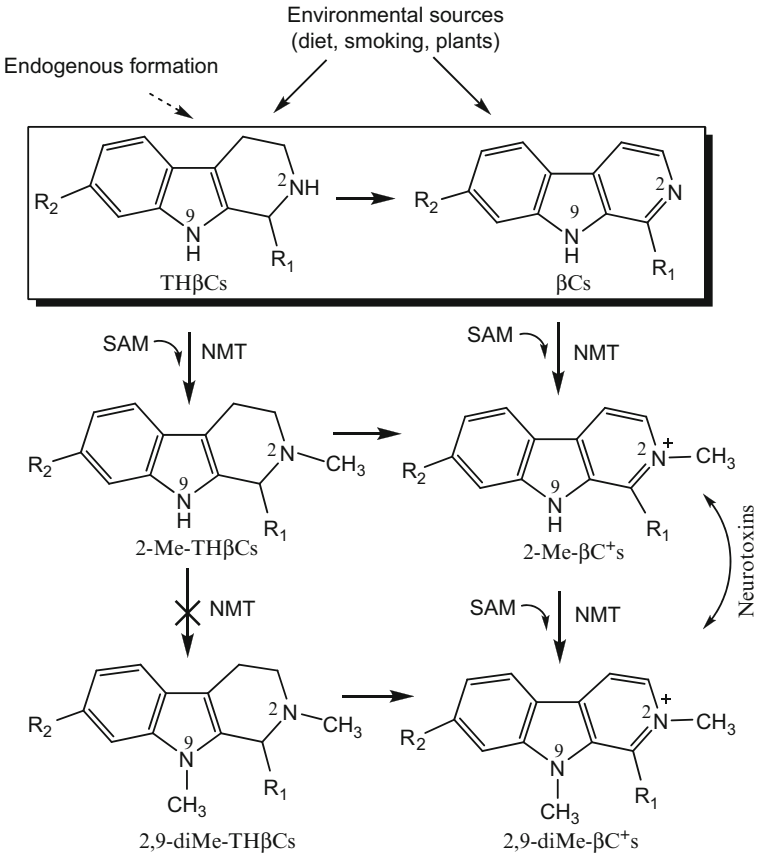

Fig. 5.3 Possible metabolic bioactivation of environmental and endogenous THβCs and βCs through *S*-Adenosyl-L-methionine (SAM)-dependent *N*-methylation by *N*-methyltransferases (NMT) in mammalian brain to afford neurotoxic 2-Me-βC$^+$s and 2,9-diMe-βC$^+$s (Collins et al. 1992; Matsubara et al. 1992b). R_1 = H, norharman series; R_1 = CH_3, harman series. Harmine series (R_2 = OCH_3, R_1 = CH_3)

Parsons et al. 2003). In addition, other brain enzymes such as phenylethanolamine-*N*-methyltransferase convert βCs, such as 9-methylnorharman into 2*N*-methylated β-carbolinium cations (Gearhart et al. 2002a) and might play a role in the bioactivation processes of βCs to neurotoxicants. An increased activity of βC 9-*N*-methyltransferase was found in the frontal cortex in postmortem human brain with PD (Gearhart et al. 2000), suggesting a role for this enzyme in PD as it might lead to increased formation of toxic 2,9-diMe-βC$^+$s available for dopaminergic neuron uptake. In summary, βCs can be sequentially *N*-methylated by NMT enzymes into 2-Me-βC$^+$s and 2,9-diMe-βC$^+$s (Fig. 5.3) which are potent mitochondrial toxicant analogs of the Parkinsonian neurotoxin MPP$^+$.

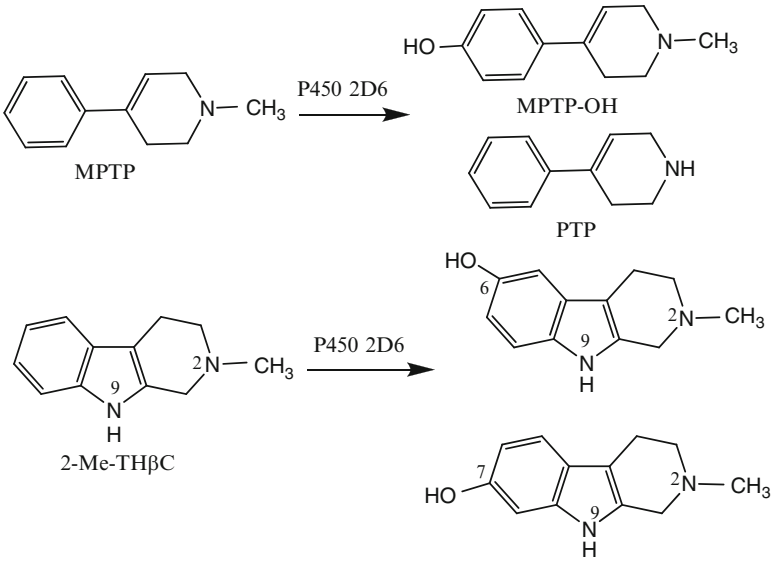

Fig. 5.4 Metabolic biotransformation (detoxification) of MPTP neurotoxin and its *N*-methyl-THβCs analogs by human cytochrome P450 2D6 (Herraiz et al. 2006)

5.3.2 Oxidative Metabolism of MPTP and Its β-Carboline Analogs

The metabolic outcome of MPTP and βCs greatly affects their neurotoxic actions. MPTP is metabolized in the liver and extrahepatic tissues by flavin monooxygenase (FMO) and cytochrome P450 enzymes 2D6 and 1A2 (Coleman et al. 1996) to give MPTP-*N*-oxide, PTP (N-demethylation) and *p*-hydroxyMPTP (*p*-hydroxylation) (Herraiz et al. 2006) (Fig. 5.4). The metabolic route by P450 2D6 is a potential route for detoxification of MPTP neurotoxin in the human liver and brain and may compete with its oxidation by MAO to neurotoxicant pyridinium cations (bioactivation route) (Herraiz et al. 2006). P450 2D6 is expressed in the liver and brain and participates in the metabolism of centrally acting drugs and endogenous neurochemicals. The presence of P450 2D6 in the brain where its expression is high in some regions (e.g., substantia nigra) may result in altered localization and inactivation of neurotoxins (Mann et al. 2008). In humans, P450 2D6 expression is highly polymorphic with poor, intermediate, extensive, and ultrarapid metabolizers, and it has been epidemiologically related with PD in poor metabolizers (Elbaz et al. 2004). Polymorphic variants of human P450 2D6 (2D6*1 and 2D6*10 allelic variants) highly differ in the metabolism of MPTP neurotoxin to PTP and *p*-hydroxyMPTP (Herraiz et al. 2006), whereas overexpression of 2D6 in neuronal cells may be neuroprotective against MPP^+ (Matoh et al. 2003; Mann and Tyndale 2010). As occur with MPTP, THβCs are also metabolized by cytochrome P450 enzymes (Herraiz et al. 2006). *N*-Methylated THβCs (e.g., *N*(2)-methyl-1,2,3,4-tetrahydro-β-carboline), which are

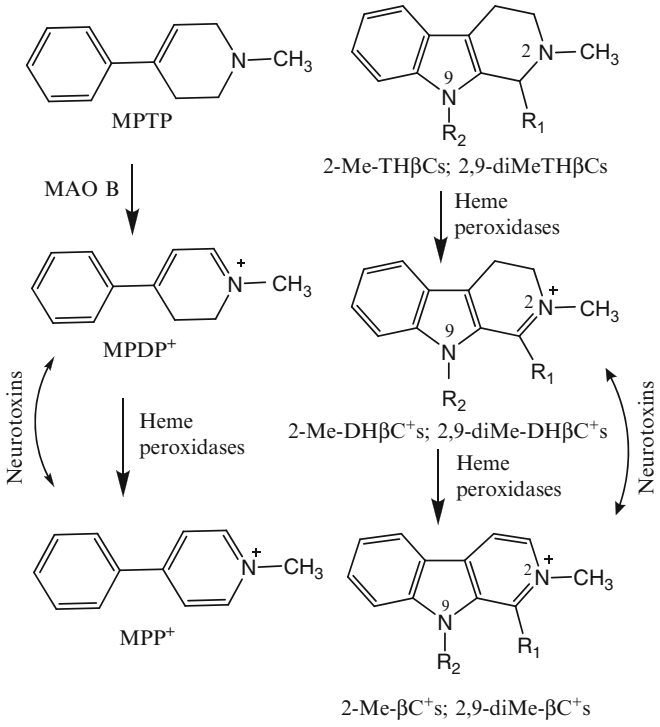

Fig. 5.5 Possible bioactivation of environmental and endogenous THβC analogs of MPTP (e.g., 2-Me-THβCs or 2,9-diMe-THβCs) by heme peroxidases to afford neurotoxic 2-Me-βC$^+$s and 2,9-diMe-βC$^+$s (Herraiz et al. 2007). MPDP$^+$ is converted by the same enzymes to MPP$^+$. R_1 = H, norharman series; R_1 = CH_3, harman series. R_2 = H or CH_3

close analogs of MPTP neurotoxin, are metabolized by P450 2D6 to 6-hydroxy and 7-hydroxy derivatives (Fig. 5.4). Therefore, P450 2D6 participates in the detoxification of *N*-methylTHβCs by an active hydroxylation pathway. The P450 2D6*1 enzymatic variant exhibited much higher biotransformation of 2-Me-THβC than the 2D6*10 defective variant, highlighting the importance of P450 2D6 polymorphism in the oxidation of these protoxins (Herraiz et al. 2006). Taken together, these results indicate that P450 2D6 play an important role in the metabolic outcome of both MPTP and βCs, suggesting a possible role for this cytochrome in neuroprotection against neurotoxins.

In contrast to MPTP, their *N*-methylated THβCs analogs, 2-methyl-1,2,3,4-tetrahydro-β-carboline (2-Me-THβC) and 2,9-dimethyl-1,2,3,4-tetrahydro-β-carboline (2,9-diMe-THβC) are not oxidized to toxic βC$^+$s cations with the participation of MAO enzymes (Herraiz et al. 2007). Neither, cytochrome P450 enzymes were able to carry out this aromatic oxidation in a significant manner. Interestingly, however, they were oxidized to 2-methyl-3,4-dihydro-β-carbolinium cations (2-Me-DHβC$^+$, 2,9-diMe-DHβC$^+$) as major metabolites and detectable amounts of 2-methyl-β-carbolinium cations (2-Me-βC$^+$, 2,9-diMe-βC$^+$) by different heme peroxidases including human myeloperoxidase and lactoperoxidase (Fig. 5.5). These results

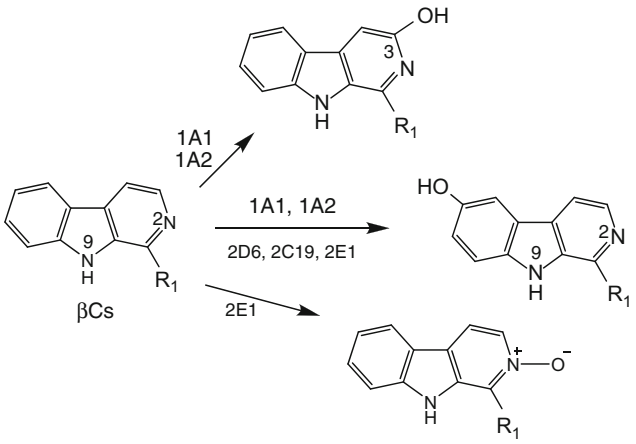

Fig. 5.6 Metabolic oxidation of neutral aromatic βCs by human cytochrome P450 enzymes. R_1 = H, norharman; R_1 = CH_3, harman (Herraiz et al. 2008)

suggest a possible role for this class of oxidoreductases in the toxic bioactivation of *N*-methylTHβCs proneurotoxins. It is noticeable that $MPDP^+$, the direct metabolite of MPTP from MAO oxidation, was enzymatically oxidized to the directly acting MPP^+ neurotoxin by the same peroxidases (Herraiz et al. 2007).

Metabolic and kinetic studies using human P450 enzymes and human liver microsomes (HLM) showed that neutral βCs (norharman and harman) are efficiently oxidized to several ring-hydroxylated and *N*-oxidation products. This oxidative metabolism was done by P450s 1A2 and 1A1 but also with the participation of 2D6, 2E1, 2C19 (Herraiz et al. 2008) (Fig. 5.6). 6-Hydroxy-β-carboline (6-hydroxynorharman and 6-hydroxyharman) was a major metabolite efficiently produced by P450 1A1 and 1A2 and to a minor extent by P450 2D6, 2C19 and 2E1. 3-Hydroxy-β-carboline (3-hydroxynorharman and 3-hydroxyharman) was produced by P450 1A1 and 1A2, whereas β-carboline-*N*(2)-oxide (harman-2-oxide and norharman-2-oxide) was produced by P450 2E1. Although *N*-demethylation (detoxification) of *N*-methylated β-carbolines and MPP^+ was not significantly catalyzed by P450 2D6 or a mixture of human P450s, several of those β-carbolines were hydroxylated to hydroxy-β-carbolines (Herraiz et al. 2006). Methoxylated βCs such as harmine and harmaline were also substrates of P450 2D6 (Yu et al. 2003a, b). These results suggest that individual variations in the levels, localization and activities of cytochrome P450 may highly change the biotransformation of βC alkaloids affecting their biological and toxicological actions. Of further toxicological interest is the differential expression of polymorphic P450 2D6 in human brain as well as the induction of P450s such as 1A1 by βCs reported recently (Mann et al. 2008; El Gendy and El-Kadi 2010). Metabolism of βCs in the liver and peripheral tissues by P450 enzymes may surely serve a detoxification route because it may divert those compounds from its *N*-methylation to neurotoxins. Nevertheless, it may also offer an alternative bioactivation route to potentially novel neurotoxic

compounds since those hydroxylated βCs produced during βC metabolism are toxic to cells (Wernicke et al. 2007; Schott et al. 2006). Further studies are needed to clarify those questions.

5.4 Neurotoxicity of β-Carbolines

A characteristic feature of neutral aromatic βCs is their selective retention in several regions of the brain. Higher levels of norharman and harman have been detected in the human pigmented substantia nigra than in the cortex (Matsubara et al. 1993). These βCs bind with high affinity to melanin and exhibit long-term retention in brain pigmented tissues of mice and frogs (a species having neuromelanin) (Östergren et al. 2004). Harman and norharman accumulate in the adrenal glands of rats and throughout the brain with much higher levels appearing in the brain than plasma (Rommelspacher et al. 1994; Anderson et al. 2006; Fekkes and Bode 1993). Accumulation of βCs in the brain may increase cell stress and apoptosis and induce neurotoxicity (Östergren et al. 2007; Yang et al. 2008). Exposure to neutral βCs produces neurotoxic features in nonprimate animals (Matsubara et al. 1998a). High doses of norharman injected in C57BL/6 mice (3 and 10 mg/kg) twice per day for five consecutive days induced motoric impairment and glial activation in substantia nigra although the number of tyrosine hydroxylase positive cells were unchanged (Östergren et al. 2006). Long-term exposure to norharman in rats (0.1-1 mg/kg) exacerbates 6-hydroxydopamine-induced Parkinsonism in animals (Haghdoost-Yazdi et al. 2010). Nevertheless, the concentrations of βCs used in those studies are generally very high and may lack physiological significance. Apparently, low doses of neutral βCs may increase dopamine and perhaps exhibit protective properties (Herraiz and Chaparro 2005; Wernicke et al. 2010; Sällström Baum et al. 1996; Herraiz and Chaparro 2006; Lee et al. 2000), whereas chronic exposure and/or high doses may trigger neurotoxicicity (Matsubara et al. 1998a; Yang et al. 2008; Haghdoost-Yazdi et al. 2010; Ergene and Schoener 1993; Storch et al. 2004a).

5.4.1 Dopamine Transporter and β-Carbolines

Neurodegeneration by Parkinsonian neurotoxins such as MPTP implies a high degree of selectivity for dopaminergic neurons. The DAT which is the uptake system for dopamine also transports MPP^+ in these neurons explaining the selectivity of this neurotoxicant. Neurotoxic βCs are also transported into dopaminergic cells through the DAT. In mouse striatal synaptosomes, neutral βCs (norharman and harman) were unfavorable substrates for DAT, whereas quarternization of these compounds to give β-carbolinium cations (βC^+s) highly increased the affinity for the transporter (Storch et al. 2004a; Matsubara et al. 1998b). 2[*N*]-Methylated βC cations (2-Me-βC^+s) were transported through DAT although with lower efficiency

than dopamine or MPP^+, and showed enhanced cytotoxicity in DAT-expressing cells, suggesting a mechanism for their selective toxicity toward dopaminergic neurons (Storch et al. 2004a). Nevertheless, the requirements for cytotoxicity in DAT-expressing cells suggested a considerable difference between βCs in the transport efficiency and the toxicity of some βC cations was independent of DAT (Wernicke et al. 2007). This suggests that other ways of uptake of neurotoxic βC cations (2-Me-βC^+s and 2,9-diMe-βC^+s) might be operative in addition to DAT (Hamann et al. 2006). Once inside the cells, the βC^+s are concentrated inside the mitochondria similar to MPP^+ in a process driven by the transmembrane electrochemical gradient (negative inside, positive outside) against their concentration gradient (Ramsay et al. 1989).

5.4.2 Toxicicity of β-Carbolines In Vitro

THβCs do not cause MPP^+-like neuronal damage (Collins and Neafsey 1985; Rollema et al. 1988; Perry et al. 1986), whereas neutral βCs like harman and norharman are weak toxicants. In contrast, the cationic forms of βCs (e.g., 2-Me-βC^+) are highly cytotoxic against neuronal cells. Table 5.1 compares the toxicological features of neutral βCs (e.g., norharman), MPP^+ and βC cations (2-Me-βC^+ and 2,9-diMe-βC^+). The βC cations are potent mitochondrial toxins interfering with energy metabolism. Differential cytotoxicities of *N*-methyl-β-carbolinium analogs of MPP^+ were investigated in PC12 cells (Cobuzzi et al. 1994). The 2,9-diMe-βC^+s (2,9-diMe-norharmanium, 2,9-diMe-harmanium, and 2-Me-harmalinium) approached or surpassed the toxicity of MPP^+ and were more cytotoxic than 2-mono-*N*-methylated βC cations (Collins et al. 1992; Cobuzzi et al. 1994). Charged β-carbolines (e.g., 2,9-diMe-βC^+) accumulate in the mitochondria and inhibit complex I with similar or higher potency than MPP^+ (Albores et al. 1990; Collins et al. 1992; Ramsay et al. 1989). Methylation in both nitrogens of βC appears to be necessary to convert a βC into a highly potent mitochondrial toxin and neurotoxicant while methoxylation in the indole ring (2-Me-harmine cation) increases cytotoxicity (Wernicke et al. 2007; Collins and Neafsey 1998). The cationic βCs are less selective for dopaminergic neurones than MPP^+ because of their lower affinity for DAT, and as a result, dimethylated β-carbolines (2,9-diMe-βC^+) were 25-fold less effective as neurotoxins than MPP^+ in rat mesencephalic dopaminergic neurons (Collins and Neafsey 1998).

The neurotoxic mechanism of 2,9-dimethyl-β-carbolinium (2,9-diMe-βC^+ or 2,9-diMe-norharmanium) was investigated in primary dopaminergic culture of the mouse mesencephalon (Hamann et al. 2006) and neuroblastome cells (Pavlovic et al. 2006). 2,9-diMe-βC^+ was a potent inhibitor of mitochondrial complex I and highly cytotoxic. It decreased respiratory activity and ATP content and increased free radical production and caspase-3 activity. Its toxicity pointed to apoptosis as a primary mode of cell death although necrosis was also observed (Hamann et al. 2006). 2,9-diMe-βC^+ reached high intracellular concentrations and caused preferential death of dopaminergic neurones compared to nondopaminergic cells

Table 5.1 Neurotoxicological features of neutral βCs (e.g., norharman), MPP^+ and its $βC^+$s analogs (2-Me-$βC^+$ refer to 2-Me-norharmanium cation; 2.9-diMe-$βC^+$ refer to 2,9-diMe-norharmanium cation)

Toxicological feature	MPP^+ vs. βCs analogs	References
Inhibition of complex I and mitochondrial respiration (mitochondrial toxins)	2,9-diMe-$βC^+$ > MPP^+ ≫ 2-Me-$βC^+$ (in both norharman and harman series)	Albores et al. (1990), Collins and Neafsey (1998), and Collins et al. (1992)
Toxicity and decrease of neuronal cell viability	MPP^+ = 2,9-diMe-$βC^+$ (toxic at μM levels) > 2-Me-$βC^+$ > norharman (toxic at mM order) = 9-Me-norharman (9-Me-βC)	Hamann et al. (2006), Pavlovic et al. (2006), and Bonnet et al. (2004)
DAT transporter efficacy	MPP^+ ≫ 2,9-diMe-$βC^+$ > norharman (inactive)	Hamann et al. (2006), Storch et al. 2004a, and Matsubara et al. (1992b)
Toxicity in neuronal cell expressing-DAT (dopaminergic-like neurons)	MPP^+ > 2,9-diMe-$βC^+$ > 2-Me-$βC^+$ ≫ norharman	Storch et al. (2004a, b), Collins et al. (1996), Wernicke et al. (2007), Pavlovic et al. (2006), and Bonnet et al. (2004)
Apoptosis	2,9-diMe-$βC^+$ and MPP^+ are highly apoptotic. Norharman is weak (only at 1 mM)	Hamann et al. (2006) and Pavlovic et al. (2006)
Production of reactive oxygen species (ROS)	2,9-diMe-$βC^+$ > MPP^+ > norharman	Hamann et al. (2006) and Pavlovic et al. (2006)
Changes in mitochondrial transmembrane potential	MPP^+ > 2,9-diMe-$βC^+$ ≫ norharman	Hamann et al. (2006) and Pavlovic et al. (2006)
Inhibition of triosephosphate isomerase (TPI)	2,9-diMe-$βC^+$ > 2-Me-$βC^+$ > norharman. MPP^+ and 9-Me-βC did not inhibit this enzyme (1 mM)	Bonnet et al. 2004)
Neurotoxicity in vivo (animals)	MPP^+ > 2,9-diMe-$βC^+$ > 2-Me-$βC^+$ > βC (norharman) = 9-Me-βC (9-Me-norharman)	Collins et al. (1992), Matsubara et al. (1998a), Neafsey et al. (1989), Ostergren et al. (2006), Pavlovic et al. (2006), and Neafsey et al. 1995)

although cellular uptake via DAT might not play a higher role in its toxicity (Hamann et al. 2006). The effects of 2,9-diMe-$βC^+$ on apoptotic changes and free radical production resembled those induced by MPP^+, whereas the neutral βC norharman only showed a weak potency at the very high doses, suggesting that *N*-methylation is needed for cell apoptosis (Pavlovic et al. 2006) (Table 5.1). 2,9-diMe-$βC^+$ and MPP^+ can induce permeability transition pores leading to rupture of the outer mitochondrial membrane and the release of the proapoptotic components, cyto-

chrome c and apoptosis induction factor (AIF) (Pavlovic et al. 2006). On the other hand, βC cations (2-Me-norharman cation) may interact with brain proteins being a target for neurotoxicity (Gearhart et al. 2002b; Bonnet et al. 2004). In this regard, the triosephosphate isomerase (TPI) is of particular interest (Bonnet et al. 2004). 2,9-diMe-βC$^+$ (2,9-diMe-norharmanium) is a potent inhibitor of TPI and it can interfere with glycolysis reducing neuronal cell viability and contributing to neurotoxicity (Bonnet et al. 2004). 2,9-diMe-βC$^+$ showed higher inhibitory potency on TPI than 2-Me-βC$^+$ or norharman, whereas MPP$^+$ was inactive, indicating that 2,9-dimethylation significantly increased neurotoxicity by this mechanism (Table 5.1). Finally, although THβCs are less toxic than aromatic βCs or βC cations, THβCs are converted into toxins when they are permanent cations (e.g., 2,2-diMe-THβC$^+$s) (Fig. 5.2). Then, toxic THβC cations (THβC$^+$s) might resemble aromatic βC cations (βC$^+$s) (Wernicke et al. 2007).

5.4.3 Neurotoxicity of β-Carbolines In Vivo

Initial attempts to generate neurotoxicity in animal models using βCs were done with THβCs such as 2-Me-THβC (Collins and Neafsey 1985; Perry et al. 1986). 2-Me-THβC is a naturally occurring compound found in plants and mammalian brain (Barker et al. 1981) that might be converted to cationic species in a process similar to MPTP/MPP$^+$ (Fig. 5.2). However, to become a neurotoxin, 2-Me-THβC requires a previous dehydrogenation step to give 2-Me-βC$^+$ cation and therefore very high doses were needed to induce any apparent neuronal loss after intraperitoneal treatment in mice (Collins and Neafsey 1998). In contrast, injection of the MPP$^+$-like *N*-methylnorharmanium cation (2-Me-βC$^+$) into the substantia nigra of rats produced nigral cell loss and highly decreased striatal dopamine and their metabolites, indicating neurotoxicity (Neafsey et al. 1989, 1995). 2-Me-βC$^+$ exerted appreciable neurotoxicity when administered by microdialysis into the striatum (Rollema et al. 1988) but it was several orders of magnitude less potent than MPP$^+$ following acute intranigral administration (Neafsey et al. 1989). *N*-Methyl-β-carbolinium cations (βC$^+$s) caused nigrostriatal toxicity following injections in the substantia of rats and three β-carbolinium cations produced lesions that approached that of MPP$^+$ (defined as 100%): 2,9-diMe-harman (94%), 2-Me-harmol (74%), and 2,9-diMe-norharman (57%) (Neafsey et al. 1995). Other βC cations or neutral βCs produced smaller lesions or were ineffective (e.g., 2-Me-βC$^+$s, norharman). MPP$^+$ highly reduced striatal dopamine whereas various βC cations reduced striatal dopamine in a lesser extent: 2,9-diMe-harman (37%), 2,9-diMe-norharman (42%), and 2-Me-harman (63%). When all βC cations are considered, there was a roughly lineal negative relationship (corr. coeff. −0.65) between lesion size and striatal dopamine level (Neafsey et al. 1995). This weak correlation may be due to the fact that the most cytotoxic βC cations were not highly selective toxins for dopaminergic cells in vivo when compared to MPP$^+$.

The highest neurotoxicity in vivo is generally produced by 2,9-dimethylated-β-carbolinium cations (2,9-diMe-βC⁺s). The neurotoxic potential of 2,9-diMe-norharmanium (2,9-diMe-βC⁺) was demonstrated by direct nigrostriatal injection in rats, which caused lesions in the substantia nigra and a reduction of dopamine levels in the striatum (Collins et al. 1992; Pavlovic et al. 2006; Neafsey et al. 1995; Matsubara et al. 2001). Toxicity of 2,9-diMe-norharmanium to nigrostriatal dopaminergic terminals was only an order of magnitude less toxic than MPP⁺ (Collins et al. 1992). A PD-like syndrome was induced in rats by intranigral administration of 2,9-diMe-βC⁺ that caused a significant decrease in the striatal levels of dopamine and its metabolites, accompanied by an enhancement of muscle tone and electromyographic activity (equivalent to muscle rigidity in PD) as well as a significant decrease in the total number of tyrosine hydroxylase-immunoreactive neurons and shrinkage of the substantia nigra (Lorenc-Koci et al. 2006). Relative neurotoxicity in vivo of neutral βCs, 2-Me-βC⁺, and 2,9-diMe-βC⁺ derivatives is consistent with neurotoxicity in cell cultures (Table 5.1). Under the same conditions, 2-Me-βC⁺s or neutral compounds (norharman) cause little neurotoxic effects compared with dimethylated compounds (2,9-diMe-βC⁺s) or MPP⁺. Nevertheless, repeated injection of high doses of norharman, 2-Me-norharmanium (2-Me-βC⁺), or 9-Me-norharman to mice decreased motor activity and produced Parkinsonian deficits (Matsubara et al. 1998b; Östergren et al. 2006). The numbers of tyrosine hydroxylase (TH)-positive cells in the substantia nigra pars compacta of norharman and 9-Me-norharman-treated mice diminished to 76 and 66% of the values in control mice, respectively (Matsubara et al. 1998a). These results suggest that neutral βCs (e.g., norharman) might induce nigrostriatal toxicity probably after their bioactivation by *N*-methylation when administered in high doses (Collins et al. 1992; Matsubara et al. 1995). Chemically, 2-Me-βC⁺ may form neutral anhydrobases after dissociation of the proton in 9-position and thereby it can cross the biological barriers and reach the brain in a way resembling neutral βCs (e.g., norharman, 9-Me-norharman). However, both neutral and 2-Me-βC⁺ are less concentrated than 2,9-diMe-βC⁺s in cell mitochondria where the electrochemical gradient is in place and consequently are less cytotoxic.

5.4.4 Tetrahydro-β-Carbolines and Halogenated THβCs as Neurotoxins

In contrast to THβCs, 2,2-dimethyltetrahydro-β-carboline cations (THβC⁺s) having a quaternary nitrogen were highly cytotoxic to DAT-expressing cells (Wernicke et al. 2007) and their toxic potency did not differ much from aromatic βC cations. In order to become neurotoxicants, THβCs and *N*-methylTHβCs may be converted into permanent βC cations by *N*-methylation or aromatic oxidation (Herraiz et al. 2007) (Figs. 5.2, 5.3, and 5.5). More attention is given to halogenated THβCs such as 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) (Fig. 5.2). TaClo may

originate in the human body from a Pictet–Spengler condensation of tryptamine and the hypnotic agent chloral hydrate (trichloroacetaldehyde) (Bringmann et al. 1996). Chloral hydrate is also a metabolite of trichloroethylene, a common industrial solvent. TaClo is permeable through the blood–brain barrier and induce PD-like symptoms in rats (Riederer et al. 2002). Similar to MPP^+ and βC^+s, TaClo inhibits the electron transfer of respiratory chain at complex I (Janetzky et al. 1995), is toxic to PC12 cells (Bringmann et al. 2006), induces apoptosis in human neuroblastome cells (Riederer et al. 2002; Akundi et al. 2004), and affects catecholamine biosynthesis by inhibiting tyrosine hydroxylase (Bringmann et al. 2002). Chloral-derived β-carbolines are not specific neurotoxicants toward dopaminergic cells (Riederer et al. 2002; Storch et al. 2006) and in contrast to MPP^+ (and βC^+s), they are highly lipophilic crossing neuronal membranes and mitochondria by passive diffusion. However, TaClo was not detected in human plasma after therapeutic administration of chloral hydrate questioning its endogenous formation (Leuschner et al. 1998).

5.5 Environmental and Endogenous β-Carbolines

Humans are continuously exposed to βCs and their precursors through the diet and tobacco smoke. Foods contain THβCs resulting from a Pictet–Spengler condensation between indolethylamines/indoleamino acids and aldehydes or α-ketoacids (Fig. 5.7) (Herraiz 2004b). Condensations arising from tryptophan afford tetrahydro-β-carboline-3-COOH (THβC-3-COOH) whereas tryptamine and serotonin afford THβCs and 6-hydroxytetrahydro-β-carbolines (6-OH-THβCs), respectively. Reactions of tryptophan with glucose provide carbohydrate-derived THβCs and phenolic aldehydes give phenolic-derived THβCs. THβCs appear in many foods from different origins and processing conditions (vegetable, animal, or fermented origin) (for a review see Herraiz 2008). THβC-3-COOH are the main class of THβCs in foods reaching up to hundreds of mg/kg whereas THβCs and 6-OH-THβCs may reach up to 30 and 4 mg/kg, respectively (Herraiz 1996; Herraiz and Galisteo 2003; Gutsche and Herderich 1997; Herraiz and Papavergou 2004). As suggested from the concentration ranges, the content of THβCs in commercial foods shows a large variability. Nevertheless, human exposure to THβCs through the diet can easily reach the level of several mg/person/day. The fully aromatic βCs norharman and harman also occur in many foodstuffs (Fig. 5.7). With a few exceptions, the level of these βCs in foods is low (i.e., in the low μg/L or ng/g order) with processed foods containing the highest amount (Herraiz 2004a, 2008). The highest occurrence of norharman and harman was found in brewed coffee (Herraiz 2002) and raisins (Herraiz 2007). Variable levels of harman were found in fermented products and seasonings. Norharman appears in toasted breads, breakfast cereals, and cookies whereas both norharman and harman occur in "well-done" cooked fish and meats (level up to 160 ng/g) (Herraiz 2004a). Although the level of aromatic βCs in foodstuffs is rather low compared to THβCs, the successive ingestion of foodstuffs containing these compounds will raise substantially the exposure to these substances.

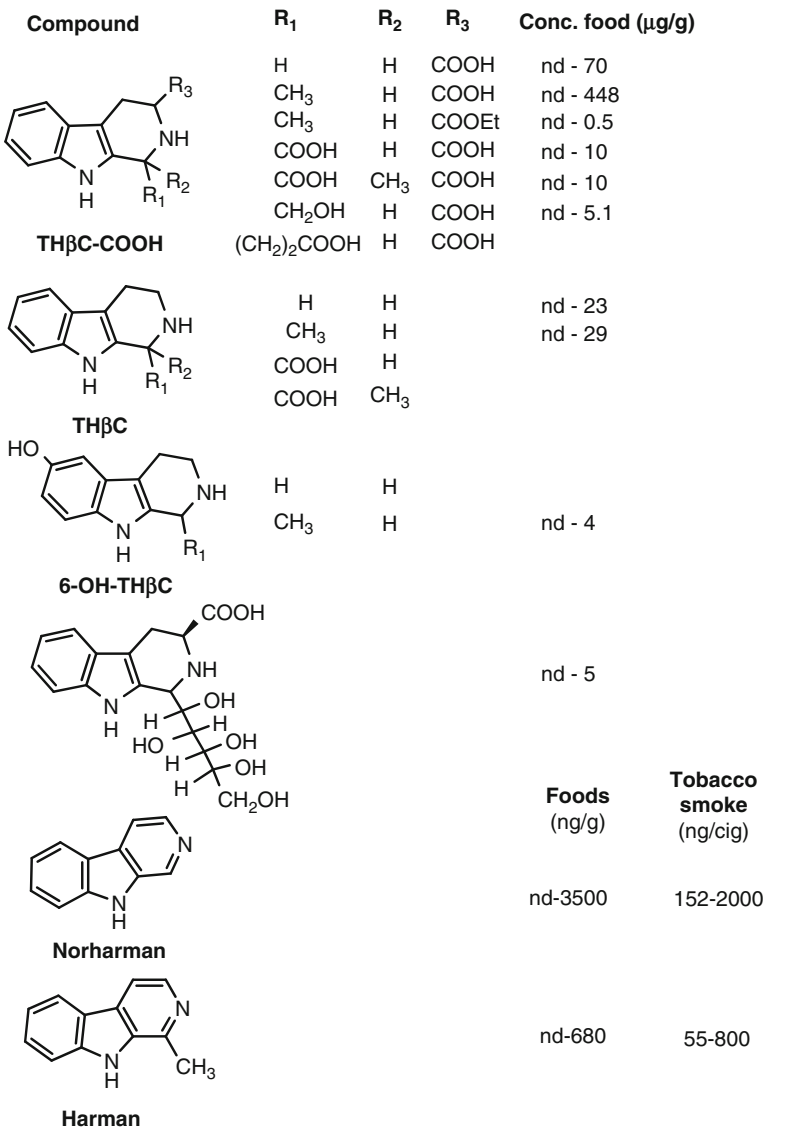

Fig. 5.7 Environmental THβCs and βCs and their concentration in foods and tobacco smoke. Under the limit of detection (nd) (for review see Herraiz 2008)

Besides foods, smoking is another important source of human exposure to aromatic βCs. Harman and norharman are present in relative high concentration in cigarette smoke (from 200 to 2,800 ng/cigarette) Herraiz 2004a. Human exposure to neutral and fully aromatic βCs may reach the level of hundreds or even thousands of μg/person/day (Herraiz 2004a, 2008). On the other hand, exposure to βCs also occurs through the ingestion of edible and medicinal plants. Two examples are *P. harmala*

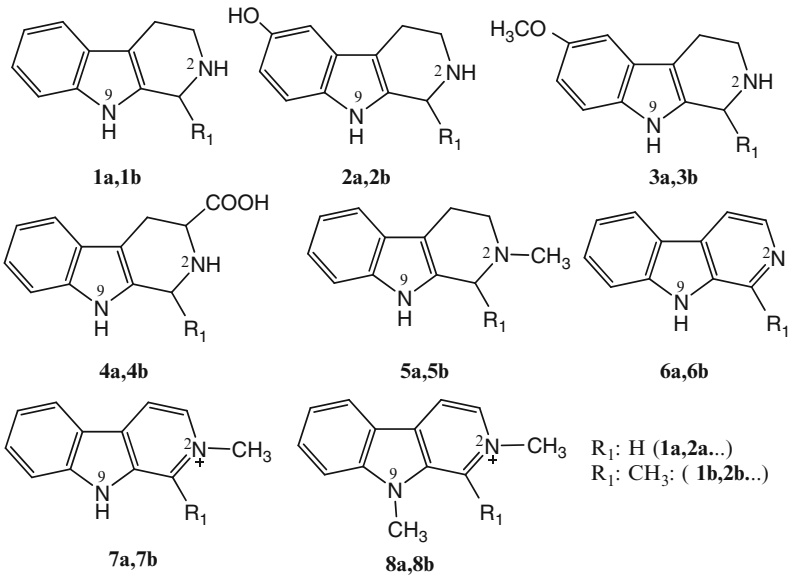

Fig. 5.8 Endogenous THβCs and βCs including neurotoxic βC cations (2-Me-βC$^+$s and 2,9-diMe-βC$^+$s) detected in mammalian fluids, tissues, and brain. R_1 = H, norharman series (**a**); R_1 = CH_3, harman series (**b**)

and *B. caapi*. These plants contain high levels of harmaline, harmine, tetrahydroharmine, harmol and harmalol which in *P. harmala* seeds reach up to 10% w/w (Herraiz et al. 2010). They are employed for medical uses and increasingly used for recreational purposes as psychoactive analogs of Ayahuasca beverage. Ingestion of these preparations may produce toxicological and neurotoxicological effects (Herraiz et al. 2010). Taken together, these results indicate that exposure to xenobiotic βCs (foods, smoking) increases the endogenous presence of βCs in humans (Herraiz 2004a, b, 2008; Spijkerman et al. 2002; Kuhn et al. 1995a; Pfau and Skog 2004; Tsuchiya et al. 1994; Rommelspacher et al. 2002).

A number of THβCs and βCs have been found endogenously in mammalian fluids and tissues (Fig. 5.8). Environmental sources (i.e., food, smoking) may contribute to this presence. In addition, βCs may form endogenously by a chemical or enzymatic cyclization of indoleamines with aldehydes or α-ketoacids (Rommelspacher et al. 1991a; Airaksinen and Kari 1981b). Briefly, tetrahydronorharman (1,2,3,4-tetrahydro-β-carboline) **1a** occurs in human urine, platelets and plasma, and rat brain (Robinson et al. 2003; Airaksinen and Kari 1981b; Tsuchiya et al. 1994; Musshoff et al. 1996; Honecker and Rommelspacher 1978); tetrahydroharman (1-methyl-THβC) **1b** in rat brain and urine, and in human platelets, plasma and urine (Tsuchiya et al. 1994; Musshoff et al. 1996; Rommelspacher et al. 1980); 6-hydroxytetrahydroharman **2b** in human and cat urine and rat brain (Musshoff et al. 1996; Beck et al. 1986), 6-hydroxytetrahydronorharman **2a** in human urine and rat brain and platelets (Musshoff et al. 1996; Rommelspacher et al. 1979), pinoline **3a** in rat brain and adrenal gland, and in human

pineal gland (Robinson et al. 2003; Airaksinen and Kari 1981b); 1-methyl-6-methoxyTHβC **3b** in rat urine (Robinson et al. 2003); 1-methyl-THβC-3-COOH **4b** in human urine and lens and rat brain (Manabe et al. 1996; Fukushima et al. 1992; Adachi et al. 1991); 2-methyl-THβC **5a** in rat brain (Barker et al. 1981); the βCs norharman **6a** and harman **6b** were detected in rat urine, plasma, arcuate nucleus and brain, bovine lung, and in human platelets, urine, plasma, and brain (Rommelspacher et al. 1991a, 1994; Parker et al. 2004; Matsubara et al. 1998a; Kuhn et al. 1995a, 1996; Fekkes et al. 1992). Endogenous βCs have been correlated with several pathological conditions in humans. Thus, elevated plasma levels of βCs (norharman and harman) have been found in alcoholics (Rommelspacher et al. 1991 b), whereas harman has been correlated with essential tremor (Louis et al. 2002). Plasma levels of norharman were significantly higher in PD patients compared to controls and although harman was also elevated its difference was not significant (Kuhn et al. 1995b). The levels of these βCs were also significantly higher in CSF of PD patients than in patients without neurological diseases (Kuhn et al. 1996). These results may suggest a possible role of harman and norharman in the pathophysiological processes initiating neurodegenerative diseases.

Neutral βCs coming from the diet or formed endogenously could be precursors of neurotoxic *N*-methylated βCs as they can be sequentially *N*-methylated to 2-Me-βC$^+$s and 2,9-diMe-βC$^+$s (Fig. 5.3). Interestingly, an increase of 2,9-diMe-βC$^+$ (2,9-diMe-norharmanium cation) was reported in the cerebrum of mice after systemic treatment with the 2,9-diMeβC$^+$ precursors, norharman, 9-Me-βC (9-Me-norharman), and 2-Me-βC$^+$ (2-Me-norharmanium cation), suggesting that simple neutral βCs may induce toxicity via their *N*-methylation (Matsubara et al. 1995, 1998a). *N*-Methylated βC cations **7a,b 8a,b** (Fig. 5.8) have been found in human brains taken during forensic autopsies and their presence considered a possible causative factor in the pathogenesis of PD. Higher concentrations of 2-methyl-β-carbolinium **7a** and 2,9-dimethyl-β-carbolinium **8a** cations were found in the substantia nigra than in the cortex (0.77 and 0.1 pmol/g, respectively, for **8a**) in control brains (Matsubara et al. 1993). In the cortex, 2-Me-norharmanium ion **7a** and 2,9-diMe-norharmanium ion **8a** were detected in almost all samples while 2-Me-harmanium ion **7b** and 2,9-diMe-harmanium ion **8b** were detectable in only two samples. Their neutral βC precursors, norharman **6a** and harman **6b** were also present in almost all samples and their levels were significantly higher in the nigra than in the cortex (16 and 0.58 pmol/g, respectively, for norharman) Matsubara et al. (1993). Moreover, *N*-methylation in vitro of the 2[β] and 9[indole] nitrogens was measured both in the cortex and in the nigra by using 9-methylnorharman and 2-methylnorharmanium cation **7a** as substrates, and the 2[β]-*N*-methylation activity was significantly higher than 9[indole]-*N*-methylation activity in both regions (Gearhart et al. 1997; Matsubara et al. 1993). Detectable levels of 2,9-diMe-norharmanium cation **8a** were found in CSF in half of PD patients, but absence in controls (Matsubara et al. 1995). 2-Me-norharmanium cation 7a was found in both controls and PD patients with a slightly higher level in PD patients than controls and the total content of *N*-methylated βC$^+$ was significantly higher in PD patients than in control (Matsubara et al. 1995). The contents of the 2-Me-βC$^+$ **7a** significantly increased with the progression of PD, but the 2,9-diMe-βC$^+$ **8a** decreased

as the disease exacerbated, supporting the hypothesis that "bioactivated" βC$^+$s, especially 2,9-diMe-βC$^+$s, may be endogenous causative factors underlying PD (Matsubara et al. 1995). Future studies are needed to clarify the neurotoxic relevance of these βC cations present in human brain.

Environmental THβCs and βCs from foodstuffs and tobacco smoke (Herraiz 2004a, b) are potential precursors of neurotoxic *N*-methyl-βC cations. However, only a very small fraction if any of the βCs ingested are expected to be converted into 2-Me-βC$^+$s and 2,9-diMe-βC$^+$s in the human body and brain. From previous results in mice (Matsubara et al. 1998a), a very high i.p. doses of norharman (0.5 mmol/Kg) produced approx. 0.73 pmol/g tissue of the 2,9-diMe-βC$^+$ **8a** and 7.5 pmol/g tissue of the 2-Me-βC$^+$ **7a** in the cerebrum of mice. Human exposure to norharman and harman from the diet and smoking will be much lower and the level of toxic βC cations produced might be probably very low to induce significant neurotoxicity. Aromatic βCs are relatively high in smoke and coffee which are associated with a reduced incidence of PD in epidemiological studies and this is against a direct role of these exogenous βCs in the pathogenesis of PD. Nonetheless, a long-term exposure to βCs (exogenous or endogenous) might still play a role in neurodegeneration after their brain accumulation and conversion to βC cations in predisposed and susceptible subjects. In fact, *N*-methylated βC cations were found in human brain reaching concentrations of 0.1–3.1 pmol/g tissue, and *N*-methylation activity of βCs was also detected (Gearhart et al. 1997; Matsubara et al. 1993, 1995). Administration of high doses of neutral βCs led to neurotoxicity (Matsubara et al. 1998a; Ostergren et al. 2006) and βCs (norharman, harman, and 2-Me-norharmanium cation) accumulated (20-fold) preferentially in the substantia nigra compared with the cortex, suggesting a possible role for these compounds as selective nigral toxins even in the absence of high affinity for DAT (Östergren et al. 2004).

In summary, neutral βCs may act as putative neurotoxins through their bioactivation into neurotoxicant pyridinium cations. Alternatively, a number of evidences suggest that neutral βCs may also exert protective actions. Thus, THβCs and βCs are antioxidants, radical scavengers, and MAO inhibitors (Herraiz 2008; Lee et al. 2000; Herraiz and Galisteo 2004). Interestingly, 9-methyl-β-carboline (9-Me-norharman) at low doses exerts neurostimulatory, neuroprotective, neuroregenerative, and anti-inflammatory effects in primary dopaminergic culture (Wernicke et al. 2010; Hamann et al. 2008). These results suggest that while exposure to low doses of βC cations produces neurotoxicity, neutral βCs and particularly 9-methylnorharman may exert protective actions. Contradictory results between toxic and protective effects may be explained by the different doses of βCs employed in the studies.

5.6 Concluding Remarks and Future Directions

The discovery of MPTP as a neurotoxin causing idiopathic PD has encouraged the hypothesis that environmental substances are causative agents in PD (Di Monte et al. 2002). A search for structural and functional analogs of MPTP has led to naturally

occurring βC alkaloids. These compounds are converted into βC cations (βC$^+$s) which are neurotoxins resembling MPP$^+$. The following conclusions can be drawn:

1. Humans are daily exposed to environmental THβCs and βCs coming from the diet and other sources (tobacco smoke, edible and medicinal plants). It is currently unknown whether dietary exposure to THβCs and βCs has neurotoxicological significance.
2. A number of THβCs and βCs, including neurotoxic βC cations occur endogenously in mammalian tissues, including the human brain. This presence might be affected by environmental exposure and/or be related with pathological conditions.
3. High doses of neutral βCs (norharman, harman, 9-methylnorharman) induce several neurotoxicological features in animals. In contrast, low doses might lack neurotoxicity and exert protective actions.
4. THβCs and βCs are bioactivated into *N*-methyl-β-carbolinium cations (βC$^+$s) by NMTs. These activities have been found in animal and human brain. A sequential *N*-methylation at the pyrido and indole nitrogens affords the highly neurotoxicants 2,9-diMe-βC$^+$s.
5. THβCs and βCs are oxidized and biotransformed by cytochrome P450 enzymes and other oxidoreductases affording new bioactive molecules such as hydroxylated and aromatic βCs. These enzymes play an important role in the outcome of the biological and toxicological effects of THβCs and βCs. Metabolic inactivation prevents the compounds from conversion into neurotoxic substances.
6. *N*-Methylated β-carbolinium cations (βC$^+$s) can be uptaken by DAT into dopaminergic cells although with less efficiency than MPP$^+$. Their selectivity for dopaminergic neurons is lower than MPP$^+$ but other routes of uptake can work for βC cations.
7. *N*-Methyl-βC$^+$s (2-Me-βC$^+$s and 2,9-diMe-βC$^+$s) are neurotoxic in vitro and cause neuronal cell death by inhibition of mitochondrial respiration and glycolysis, oxygen radical production, and apoptosis. Potencies of dimethylated βC compounds (2,9-diMe-βC$^+$s) as mitochondrial toxins and apoptotic agents approach that of MPP$^+$ and were higher than 2-Me-βC$^+$s. 2,2-diMe-THβC cations (THβC$^+$s) are also toxic to cells and might resemble aromatic βC cations.
8. Direct exposure of the brain to *N*-methylated β-carbolinium cations (2,9-diMe-βC$^+$s) produces neurotoxicity in animals. These βC$^+$s have been detected in the human brain.

In summary, a long-term exposure to xenobiotic or endogenous THβCs and βCs may induce a neurotoxicological risk in humans as these compounds can be bioactivated into putative toxins (βC cations) that are trapped and accumulated in the brain. Nevertheless, most of the studies performed so far were studies in vitro and using high doses of βCs (neutral βCs) or directly applying βC cations in brain. Then, their physiological significance regarding actual human exposure to THβCs and βCs is questionable. The toxicological risk of β-carbolines is affected by key factors such as their bioactivation by NMTs and their metabolic biotransformation by cytochrome P450 enzymes and other oxidoreductases. Only a very small fraction

of exogenous or endogenous βCs if any is expected to be converted into putative toxic *N*-methylβC cations in the brain. Future studies are warranted to highlight the importance of metabolism in βC toxicity, including individual P450 polymorphism and induction. Studying the possible epidemiological associations between exogenous and endogenous βCs and neurodegenerative diseases as well as the assessment of the presence of βCs and neurotoxicant βC cations in the human brain of patients in relation to different stages of disease would be of much interest to estimate the neurotoxicological significance of βC compounds. Noticeably, βCs are bioactive substances in many targets and these alkaloids may also exert neuroprotective actions (see other chapters) suggesting that still unknown factors may delimitate or define whether these substances function as neuroprotectants or neurotoxicants.

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Chapter 6
β-Carbolines: Occurrence, Biosynthesis, and Biodegradation

Hans Rommelspacher, Catrin Wernicke, and Jochen Lehmann

Contents

Abstract Studies clearly demonstrated biosynthesis of β-carbolines (βCs) in animals and humans. Precursor compounds include serotonin (syn. 5-hydroxytryptamine), tryptamine, and tryptophan with either acetaldehyde or pyruvate as cosubstrates. βCs are metabolized efficiently. Alcohol consumption and smoking affect their biosynthesis and biodegradation. Alcohol consumption increases the biosynthesis of harman (1-me-βC) which induces voluntary alcohol intake possibly by increasing the activity of dopamine neurons of the mesolimbic system (see Chap. 10). On the

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other hand, smoking induces the biodegradation of βCs. Norharman-2-N-oxide, a metabolite of norharman (βC) in brain and liver protects norharman from methylation to neurotoxic βCs. Furthermore, βCs form complexes with acetic acid and other hydrogen donors which may prevent the N-methylation to the toxic quaternary cations. Progesterone binding to CYP17 is completely blocked by norharman in contrast to harman which indicates inhibition of androgen biosynthesis. These findings point to multiple functions and interactions of βCs some of which will be presented in more detail in subsequent chapters.

Keywords Biosynthesis • Tryptophan • Tryptamine • 5-Hydroxytryptamine • Pyruvate • Acetaldehyde • Biodegradation • Cytochrome • Alcohol • Smoking • Norhaman-N-oxide • Complex with acetic acid • Androgen

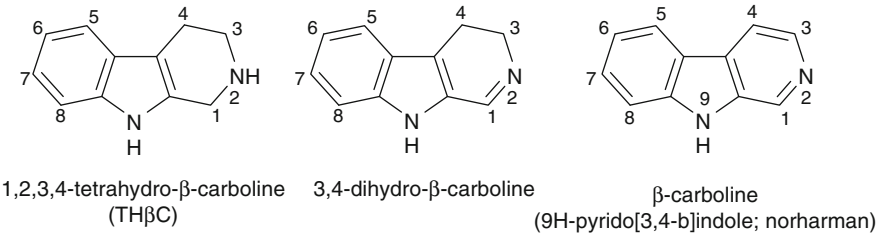
6.1 Introduction

β-carbolines (βCs) have been identified and characterized from a variety of mammalian tissues in both in vitro and in vivo studies (Wyatt et al. 1975; Hsu and Mandell 1975; Rommelspacher et al. 1976; Honecker and Rommelspacher 1978; Shoemaker et al. 1978; Airaksinen and Kari 1981a; Baker et al. 1981). Several reviews have been published since the first wave of publications in the 1970s reporting the evidence for the natural occurrence of the βCs in plants, animals, and humans (Deitrich and Erwin 1980; Airaksinen and Kari 1981a,b; Collins 1985; Rommelspacher and Susilo 1985; Rommelspacher et al. 1991; Brossi 1993; Pfau and Skog 2004; Cao et al. 2007; Abramovitch and Spenser 1964) the latter review focuses on the interaction of βCs with DNA and antitumor, antiviral, antimicrobial, antiparasitic, and antithrombotic activities). To introduce in the topic, selected aspects will be presented here before reporting the evidence for the neuroprotective and neurorestorative properties of some βCs.

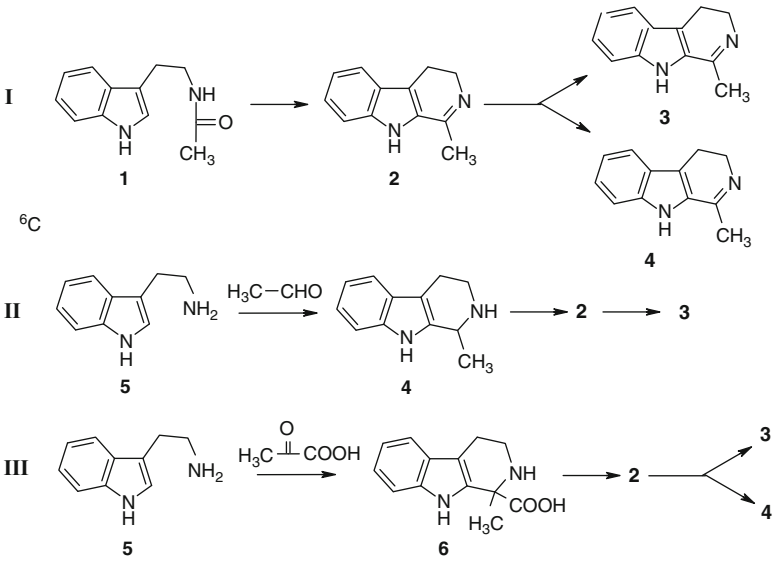
6.2 Biosynthesis

1-Unsubstituted βCs: The in vitro and in vivo formations of the compounds 1,2,3,4-tetrahydro-β-carboline (THβC), 3,4-dihydro-β-carboline, and the fully aromatic βC 9*H*-pyrido[3,4-*b*]indole (trivial name: norharman) were investigated by administration of possible precursors (Scheme 6.1).

The studies revealed that the biosynthesis involves a carbon unit transfer from 5-methyl-tetrahydrofolate (MTHF) to tryptamine followed by a cyclization reaction yielding THβC (Lauwers et al. 1975). The other βCs depicted above (Scheme 6.1) are formed consecutively by oxidation (=dehydrogenation). It is not clear whether the oxidation steps involve enzymes in vivo (Barchas et al. 1974; Wyatt et al. 1975;



Scheme 6.1 Structures of some βCs



Scheme 6.2 Biosynthetic pathways of βCs

Mandel et al. 1974; Hsu and Mandell 1975; Rommelspacher et al. 1976). To investigate whether also tryptophan serves as a precursor, smokers and nonsmokers received an oral tryptophan load and both tryptophan as well as norharman (formula: Scheme 6.1) were determined in plasma. The levels of both compounds were elevated in the individuals who received tryptophan. The levels of norharman in smokers were higher under control and smoking conditions. The authors calculated that tryptophan contributes to 26% of the plasma levels of norharman (Fekkes et al. 2001).

Substituted βCs: The biosynthesis of 1-methyl-βCs may proceed in mammals by three different modes of cyclodehydration shown in Scheme 6.2. Kveder and Mc Issac (1961) detected trace amounts of 6-methoxyharmalan in the urine of rats after administration of [^{14}C]melatonin (N-acetyl-6-methoxytryptamine) suggesting a Bischler–Napieralski mode of cyclization (Scheme 6.2 route I). It is notable that under in vitro conditions both nitrogens of βCs are complexed by acetic acid (Reyman et al. 2007, see end of chapter biodegradation for details).

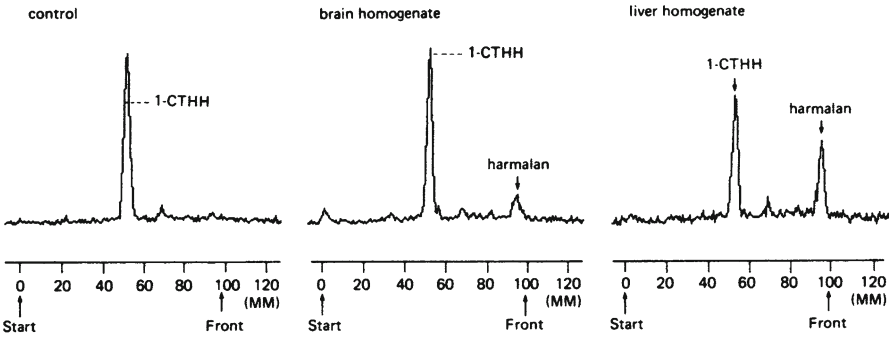


Fig. 6.1 In vitro decarboxylation of [^{3}H]tetrahydroharman-1-carboxylic acid. Radiochromatograms after thin layer chromatography separation. Boiled brain homogenate was incubated with radiolabeled 1-CTHH, control, left panel, untreated brain homogenate was incubated with radiolabeled 1-CTHH, middle panel, and liver homogenate, right panel.

The biosynthesis with aldehydes, in particular acetaldehyde, is favored by some investigators because aldehydes are very reactive agents (route II, cyclization according to Pictet–Spengler). Ethanol loading – the precursor of acetaldehyde in rats and humans increased βC concentrations in plasma, urine, and rat brain indicating that this route of formation occurs in vivo (Peura et al. 1980; Rommelspacher et al. 1980, 1984). 6-OH-THH was identified in human and cat urine with different proportions of the (S)(−)- and (R)(+)-enantiomers (Beck et al. 1986). The third route is a condensation reaction with α-ketoacids, for example, pyruvate, yielding 1-carboxy-tetrahydroharman (1-CTHH, route III, reaction according to Pictet–Spengler). Collins et al. (1982) detected 6-hydroxy-1-CTHH in the cerebrospinal fluid from monkeys indicating serotonin and pyruvate as the precursor compounds. Another study was conducted to demonstrate that this mode of formation occurred in vivo.

[^{3}H]Tryptamine and pyruvic acid were injected into the lateral cerebral ventricle of rats pretreated with an inhibitor of monoamineoxidase. [^{3}H]1-CTHH was detected in brain tissue within 5 min after application suggesting a rapid cyclodehydration reaction in vivo (Fig. 6.1). High doses of the monoamine oxidase inhibitor prevented the formation of the βC suggesting an enzymatic formation (Susilo and Rommelspacher 1987). The next step in the formation of tetrahydroharman (THH), harmalan, and harman is the decarboxylation. [4-^{14}C]1-CTHH was given to rats intraperitoneally (Fig. 6.1, Susilo and Rommelspacher 1988 ; Susilo et al. 1987). A time-dependent formation of [^{14}C]THH was found in lung and spleen indicating an important role of these organs in the biosynthesis of THH. [^{14}C]harmalan was detected as the major metabolite in all tissues including brain. The amount of harman was clearly lower. The metabolism of [4-^{14}C]1-CTHH in the brain was investigated after injection into the cerebral ventricle. High levels of [^{14}C]harmalan were detected in hippocampus and hypothalamus and lower levels in other brain regions. The largest amount of [^{14}C]harman was detected in the lung, whereas far lower levels were found in all other organs (Susilo and Rommelspacher 1988). Investigations of the oxidative decarboxylation reaction were also conducted in mouse brain homogenate and compared with the reaction in buffer. The reaction was sixfold increased in brain homogenates indicating

an enzymatic catalysis (Gynther et al. 1986). These findings suggest that the in vivo formation of βCs methylated in position 1 proceeds mainly via an enzymatic oxidative decarboxylation of 1-CTHH.

6.3 Biodegradation

6.3.1 Unsubstituted βCs

In vitro experiments: Using human liver microsomes and recombinant P450 isoenzymes revealed that the aromatic βC norharman is efficiently metabolized (Herraiz and Guillén 2008). Individual isoenzymes preferred the N-oxidation and hydroxylation of specific sites of the molecule. This becomes evident by comparing the V_{max} values of experiments with liver microsomes (1.9 pmol/min and pmol P450) and recombinant P450 1A2 (115 pmol/min and pmol P450), and P450 1A1 (22 pmol/min and pmol P450) in the formation of 6-OH-norharman, the main metabolite. Other metabolites were identified as 3-OH-norharman and norharman-2-N-oxide, the latter formed by P450 2E1. Interestingly, no substituent in the 7-position was identified. The authors pointed to the fact that P450 1A1 is a highly inducible CYP, for instance by tobacco smoke (Guengerich 2005) and that the K_m for norharman was lower than that for harman. Furthermore, in contrast to P450 1A2, the P450 isoenzymes 1A1, 2D6, and 2E1 are expressed in lung and brain where βCs are likely to occur and accumulate, for example, in smokers. This means that in smokers the induced enzymes convert the βCs in the brain rapidly into the inactive metabolites notably to the 2-N-oxide which prevents the N-methylation to the neurotoxic 2-methyl-βC$^+$. These conditions may be one of the reasons that the prevalence of Parkinson's disease is lower in smokers than in the general population. This point will be addressed later in the text.

Norharman binds with high affinity to steroidogenic cytochromes CYP11 (cholesterol monooxygenase and steroid-11β-monooxygenase) and CYP17 (steroid-17α monooxygenase/steroid-C17, 20-lyase). Progesterone binding to CYP17 was competitively inhibited in contrast to harman and other βCs which were nearly ineffective (Kühn-Velten 1993). These findings implicated norharman but not harman as an inhibitor of androgen biosynthesis and possibly of other steroid hormones.

In vivo experiments: Experiments were conducted by administration of [1-^{14}C]1,2,3,4-tetrahydro-β-carboline in rats. At least six metabolites were excreted into the urine. Both 6- and 7-hydroxy-1,2,3,4-tetrahydro-β-carboline occurred in approximately equal amounts. This observation points to the possibility of an intermediate epoxidation and ringopening again. This is in contrast to the findings with human microsomes (see above). The metabolites differed between the sexes: the fraction of sulfate conjugates was larger in male than in female rats. An alternative pathway yielded norharman. Norharman was subsequently biodegradated by oxygenation in position 2 resulting in N-oxide and a further conversion via an oxaziridine to 1,2-dihydro-β-carboline-1-one. This βC was converted to 1-ol, depending

on the thermically preferred tautomer (Greiner and Rommelspacher 1984). As the nitrogen is not basic anymore, N-methylation is not possible to occur provided no respective enzyme is present in vivo.

[1-^{14}C]6-MeO-THβC was given to rats by intraperitoneal injection. The major metabolic pathways were hydroxylation in the 7-position and demethylation of the 6-methoxy group. The metabolites were excreted in the urine in nearly equal amounts, almost entirely as glucuronide and sulfate conjugates with the latter predominating. Both conjugated metabolites were also detected in the bile. At all time intervals, the level of radioactivity in the brain consisted of 85–95% of the unchanged [1-^{14}C]6-MeO-THβC (Ho et al. 1972).

In conclusion in vitro and in vivo experiments with [^{3}H]norharman demonstrated, that the βC binds preferentially to cytochrome P450 2E1 and to a lesser extent to 1A1/2. Interestingly, ethanol displaced the ligand from recombinant 2E1 microsomes in contrast to harman which was inactive. At the end of a forced treatment period of rats with ethanol, the levels of norharman in plasma were significantly elevated supporting the contention of a competition of norharman and ethanol at the level of the metabolizing enzyme (Stawowy et al. 1999).

6.3.2 Substituted βCs

In vitro experiments: The metabolism of harman was investigated in microsomes from mice pretreated either with vehicle (controls), phenobarbitone, or 3-methylcholanthrene. The latter compounds served as inducers of cytochromes. The major metabolite of harman was identified as 6-OH-harman under all conditions. Initial reaction rates for harman disappearance showed a fourfold induction by phenobarbitone and 10.6-fold by methylcholanthrene. The authors concluded that the rapid metabolization is important in detoxication because the metabolite was less active in pharmacological tests (Tweedie and Burke 1987; Tweedie et al. 1988).

Studies with human microsomes and recombinant P450 isoenzymes revealed that harman is metabolized to the 1-methyl substituted metabolites analogous to those described for norharman above (Herraiz and Guillén 2008).

The O-demethylation of harmin and harmalin by human liver microsomes and individual recombinant CYPs was investigated to assess whether CYPs could ameliorate the tremorigenic action of βCs. It had been reported that the demethylated βCs were much less tremorigenic than harmin and harmalin. CYP 2D6 catalyzed the O-demethylation of harmaline and CYP 2D6 and 1A1 that of harmin. The turnover numbers of CYP 2D6 were among the highest ever reported for CYP 2D6 substrates (Yu et al. 2003). Both CYP 1A1 and 2D6 are expressed in the brain and notably, 2D6 was elevated in alcoholics (Strobel et al. 2001; Mikays et al. 2002).

In vivo experiments: [^{3}H]Harmin was given by intraperitoneal injection to male rats. The compounds excreted in the urine were harmin and harmol both as the free

base and in addition harmol as conjugates (69% sulfate and 18% glucuronide) (Slotkin and DiStefano 1970).

Tetradeuterated 1-methyl-1,2,3,4-tetrahydro-β-carboline was orally administered to a human subject. The urine was collected and the metabolites were identified as 6-OH-1methyl-TβC and 7-OH-1methyl-TβC and their conjugation products (Tsuchiya et al. 1995).

The N-methylation of βCs is reported by Herraiz in Chap. 5 of this book.

An additional aspect should be mentioned. It has been found by NMR experiments that hydrogen bonding between βCs and two (norharmane) or three (harmane) molecules of acetic acid leads to complexes (Reyman et al. 2007) in which the pyridine nitrogen is kind of blocked by serving as the hydrogen bond acceptor. Accordingly, one could speculate that such complexations with acetic acid or other hydrogen donors may prevent the N-methylation to the toxic quaternary cations.

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Chapter 7
β-Carbolines and Neuroprotection: Inhibition of Monoamine Oxidase

Hans Rommelspacher

Contents

Abstract Recent studies with MAO B inhibitors do not support the notion of neuroprotective actions by direct inhibition of the isoenzyme nor by inhibition of MAO A. MAO activity is present in the brain in large amounts. MAO B is increased in Parkinson's disease (PD) due to gliosis and in smokers by a compensatory mechanism for the inhibition of the enzyme by compounds contained in tobacco smoke. Both isoenzymes should be inhibited to a certain extent to achieve neuroprotection by decreasing the production of detrimental reactive oxygen species (ROS). The correlation between the levels of the βCs norharman (inhibitor of MAO B) and harman (inhibitor of MAO A) in tobacco smoke and the proportion of the inhibited isoenzymes in the human brain measured by PET strongly support the notion that these two βCs are responsible for the inhibition of MAO A and B in human brain from smokers. Epidemiologic studies show that smoking subjects have a reduced risk for developing PD. Studies using in vivo microdialysis and electrophysiology demonstrate a preference of the βCs for dopamine neurons. Provided smoking a full cigarette results in more than 88% nicotinic receptor occupancy associated with

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desensitization of these receptors, we postulate that the βCs contribute substantially not only to the neuroprotective actions of smoke with a reduced risk for PD but also to the addictive effects specifically to improve mood and pleasure.

Keywords Monoamine oxidase A • Monoamine oxidase B • Norharman • Harman • Neuroprotection • Smoking • Nicotinic receptor • Parkinson's disease • Addiction

7.1 Introduction

Monoamine oxidase (MAO, EC 1.3.3.4) is localized in the outer mitochondrial membrane and catalyzes the oxidative desamination of a range of monoamines, including 5-hydroxytryptamine (5-HT, syn. serotonin), histamine, and the catecholamines dopamine (DA), noradrenaline, and adrenaline. The reaction produces hydrogen peroxide, the corresponding aldehyde, and either ammonia in the case of primary amines or a substituted amine from the secondary amines, for example, methylamine from adrenaline. Two isoenzymes of MAO (MAO A and MAO B) are present in most mammalian tissues. MAO A catalyzes the oxidation of 5-HT, whereas both isoenzymes catalyze the oxidation of dopamine, noradrenaline, adrenaline, tryptamine, and tyramine in most species. In human brain MAO B constitutes ~80% of the total MAO activity (Kornhuber et al. 1989).

The active site of MAO A consists of a hydrophobic cavity of ~550 and ~450 $Å^3$ in the human and rat enzymes, respectively. An important and unique component of the structure of the active site of hMAO A is a loop conformation which differs in that of hMAO B and rat MAO A and B. Thus, there is an intriguing difference between the active sites of human and rat MAO A and put into question the use of MAO A from nonhuman sources in drug development for use in humans. The active site of human MAO B is a hydrophobic cavity with a volume of 700 and of 490 $Å^3$ in the rat. The site is occupied by the redox-active isoalloxazine ring of the covalently bound FAD coenzyme (De Colibus et al. 2005).

7.2 Neuroprotective Actions of Conventional MAO Inhibitors

Low molecular weight materials that can act as endogenous MAO inhibitors include isoquinolines, β-carbolines, isatin, phosphatidylserine, and quinolinic acid. On the other hand, cigarette smoke is probably the main source of exogenous MAO inhibitors. Yu and Boulton (1987) reported that small compounds in tobacco smoke inhibit both isoforms of MAO. MAO A is effectively inhibited on average of 28% in human brain. By comparison, treatment with tranylcypromine (10 mg per day) for 3 days reduced MAO A activity by 58% (Fowler et al. 1996a). Brains of living smokers showed a 40% decrease in the level of MAO B relative to nonsmokers or former

smokers (Fowler et al. 1996a, b) and a significant reduction of platelet MAO B (Rommelspacher et al. 2002). Thus, smoking is associated with enhanced activity of dopamine and other monoamine neurotransmitters as well as with decreased production of hydrogen peroxide, a source of reactive oxygen species (ROS), a primary factor in neurodegeneration. These conditions may explain why lowered MAO B activity is associated with a reduced risk of Parkinson's disease (PD) in smokers (Scott et al. 2005).

Levels of MAO B are increased in the brains of patients with PD and Alzheimer's disease (AD) as a consequence of gliosis (Kennedy et al. 2003) which might contribute to oxidative stress in these disorders (Birks and Flicker 2003). There are insufficient data to conclude whether any MAO B inhibitor significantly delays disease progression in early PD and AD [neither inhibition of MAO A nor MAO B affects steady state levels of brain dopamine: only when both forms are inhibited does dopamine activity increase (Green et al. 1977)]. We will come back to this point later in this chapter.

Neurodegenerative diseases share many of the pathological features of PD and AD such as oxidative stress, iron accumulation, excitotoxicity, inflammatory processes, misfolding of toxic proteins that cannot be degraded after ubiquitination, and apoptosis. Elevated ROS production has been proposed to be a causative agent in several neurodegenerative diseases (Emerit et al. 2004; Barnham et al. 2004; Andersen 2004).

Several studies suggested that MAO B inhibitors *L*(−)deprenyl (selegiline, N-methyl-N-[(2R)-1-phenylpropan-2-yl]prop-2-yn-1-amine), rasagiline (1(R)-N-prop-2-ynyl-2,3-dihydro-1H-inden-1-amine), and other propargylamines act as neuroprotectants specifically in models of PD and that MAO B-catalyzed reactions might contribute to neurotoxicity. However, not all MAO B inhibitors are effective in neuroprotection (Ansari et al. 1993; Mytilineou 1998) and it seems that MAO inhibition might not be the principal protective mechanism shared by these compounds. The doses of *L*(−)deprenyl required were lower than those reported necessary to inhibit the enzyme (Ansari et al. 1993) and the (S)-enantiomer of rasagiline, which does not inhibit MAO B, was also protective (Huang et al. 1999). It has been suggested that the protective compounds act as antioxidants. Although the protective concentrations are too low for a direct antioxidant action to be important, they do appear to increase cellular antioxidant capacity (Mytilineou 1998).

Structure–activity studies revealed that a free propargylamine moiety with a hydrophobic structure is essential for MAO inhibition. Free propargylamine inhibits MAO A but not MAO B, suggesting that the hydrophobic moiety increases the affinity to MAO B (Yu and Davies 1999). Changes in the stereochemical properties and the ring size and alternative substitution of the hydrophobic structure affect the MAO-inhibiting activities. Furthermore, *L*(−)deprenyl and other propargylamine derivatives are generally antiapoptotic. They prevent the mitochondrial permeability transition, the decline in membrane potential and cytochrome-C release in isolated mitochondria and the decline of antiapoptotic Bcl-2 proteins (Akao et al. 2002). Rasagiline-related propargylamines even stimulated their formation in MAO-A-containing cells but not in MAO-B-containing cells, suggesting the involvement of

MAO A and not MAO B in the antiapoptotic activities (Naoi et al. 2006). These findings suggested that the dose required for induction of Bcl-2 proteins must be relatively high. Apoptosis occurs in neurodegenerative diseases, thus it was not surprising that many of the complex events were attenuated or reversed by the propargylamines (Magyar and Szende 2004). It has been suggested that a key event of antiapoptotic actions is the specific binding of propargylamine compounds to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), shown to be an important mediator of neuronal apoptosis (Kragten et al. 1998). GAPDH binding of propargylamines prevented the translocation of GAPDH together with the transcriptional activator transforming growth factor-beta-inducible early gene 2 (TIEG2) into the nucleus and secondarily dense GAPDH nuclear accumulation typical of GAPDH-associated apoptosis while allowing the enzyme to retain the glycolytic capacity. Inactivation of GAPDH decreased the synthesis of proapoptotic proteins like Bcl-2-associated X protein (BAX), cJun, prevented the TIEG2-induced MAO-B gene expression (Ou et al. 2009), and increased the synthesis of antiapoptotic proteins like Bcl-2, Cu/Zn-SOD, and heat-shock protein 70 (Tatton et al. 2002).

Although the neuroprotective activity of *L*(−)deprenyl in PD patients remains controversial possibly because its metabolite methamphetamine counteracts the neuroprotection afforded by *L*(−)deprenyl (Palhagen et al. 2006), it has antioxidant and neuroprotective effects in experimental studies (Youdim and Bakhle 2006). Its restricted derivative, rasagiline, which is different from *L*(−)deprenyl in that it is not metabolized to amphetamine and/or methamphetamine but instead to the neuroprotective metabolite aminoindan (Bar-Am et al. 2007), has been shown to be effective in early PD (Bayes et al. 2006; Parkinson study group 2004). A recent study provided evidence supporting both neuroprotective and neurorestorative activities for rasagiline in an animal model of PD. Neurodegeneration was produced by inhibition of the ubiquitin-proteasome system (UPS) by lactacystin (Zhu et al. 2008). Again, these studies confirmed, that MAO B inhibitory activity cannot explain either the neuroprotective or neurorescue properties of MAO inhibitors *L*(−)deprenyl and rasagiline. In fact, MAO B inhibitors have been reported to possibly enhance neurotoxicity by proteasome inhibition (Fornai et al. 2003). In addition optical isomers of rasagiline and *L*(−)deprenyl are devoid of MAO B inhibitory activity, but they had the same potency in neuroprotection (Youdim et al. 2001; Olanow 2006). In addition both promoted free radical scavenging by enhancing superoxide dismutase (SOD) and catalase activities (Tabakman et al. 2004). Both drugs increased production of neurotrophins such as nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and protected neurons from inflammatory processes (Nagatsu and Sawada 2006). Furthermore, as mentioned earlier in this chapter, the propargylamines bound to glyceraldehyde-3-phosphate dehydrogenase to decrease synthesis of proapoptotic proteins and increased synthesis of antiapoptotic proteins (Tatton et al. 2003).

A further mode of action of MAO inhibitors is binding to an imidazoline binding site of the enzyme, which might contribute to neuroprotection (Raddatz et al. 1995). MAO inhibitors bind to other proteins besides MAO, such as semicarbacide-sensitive amine oxidase (EC 1.4.3.4; Holt et al. 2004) and dopamine D1 and D2 receptors (Levant 2002).

7.3 Inhibition of MAO A and MAO B by β-Carbolines

The βCs inhibit monoamine oxidase. Although the findings in the previous section did not support the notion that direct inhibition of MAO A or MAO B acts as a neuroprotectant. None of the studies investigated whether inhibition of both enzymes simultaneously backs neuroprotection. The reason for considering this condition is the lack of a sufficient explanation for the well-documented fact that the prevalence of smoking is reduced in patients with Parkinson's disease compared with the general population (Baron 1986; Morens et al. 1995; Checkoway and Nelson 1999; Gorell et al. 1999). These findings strongly suggest protection of dopaminergic neurons in the brain by constituents of tobacco smoke. The neuroprotective action of tobacco smoke cannot be generalized to all types of neurons because such an association was not consistently demonstrated in Alzheimer's disease. Positron emission tomography studies showed a 40% decrease in binding capacity of the tracer substance of monoamine oxidase B in the brains of active smokers relative to nonsmokers or former smokers (Fowler et al. 1996b). An inhibitor of monoamine oxidase B, which is found in tobacco smoke in remarkably high concentrations, is norharman (synonymous with β-carboline). An amount of 12.6 μg norharman/g tobacco is present in bright cigarette smoke (Poindexter and Carpenter 1962; Totsuka et al. 1999). Norharman preferentially inhibits monoamine oxidase B ($K_i = 730$ nM, brain tissue, rats, May et al. 1991). It occurs naturally in human blood plasma (Rommelspacher et al. 1991). The highest natural concentration was found in human substantia nigra (16 nmol/kg tissue, Matsubara et al. 1993). The levels were increased in plasma from acutely smoking subjects (Breyer-Pfaff et al. 1996; Rommelspacher et al. 2002). Norharman readily crosses the blood–brain barrier and is accumulated in the brain (partition factor 3, Fekkes and Bode 1993). *L*(−)deprenyl inhibited specific [^{3}H]norharman binding to crude mitochondrial membranes from rat brain with a K_i-value of 130 nM, supporting the notion of high-affinity binding of norharman to the active site of monoamine oxidase B in brain tissue (Pawlik and Rommelspacher 1988). Harman, a methylated derivative of norharman, was also present in tobacco smoke (3.6 μg/g tobacco measured in bright cigarette smoke, Poindexter and Carpenter 1962) and inhibited monoamine oxidase A ($K_i = 220$ nM, in tissue from rat brain (May et al. 1991; Rommelspacher et al. 1994) and 55 nM in recombinant human MAO A (Herraiz and Chaparro 2005), the difference in K_i-values is explained by the difference of the human and rat binding site of MAO A, see above). Other compounds in tobacco smoke exerted lower affinity to MAO (for review see van Amsterdam et al. 2006).

Smokers had a baseline concentration of norharman of 20 nM in platelets and 28 nM after smoking two cigarettes. The respective values for harman were 6.8 and 16.2 nM. Studies of the elimination kinetics indicated that heavy smokers can easily achieve 100 to 150 nM norharman in platelets by smoking ten cigarettes within a few hours. Considering a loss of norharman during work-up of the platelets and the accumulation of the lipophilic compound in brain (Fekkes and Bode 1993) and moreover in the microenvironment of the monoamine oxidase, the contribution of

the βC to the inhibition of monoamine oxidase B in smokers amounts to approximately 30% (Rommelspacher et al. 2002), similar to the inhibition observed by positron emission tomography in human brain (40%, Fowler et al. 1996a, b). It is noteworthy, that harman occurred in tobacco smoke by about a fourth of the amount of norharman. The inhibition of MAO A, the target of harman in the brain of smokers, was 28% (Fowler et al. 1996a). These findings strongly support the notion that these two βCs contained in tobacco smoke essentially contributed to the inhibition of both isoforms of MAO in vivo.

Dopamine, the most affected neurotransmitter in PD, is metabolized by both forms of monoamine oxidase in human brain tissue. The ratio of activities vary considerably from brain region to brain region, from about 1:1 in cerebral and cerebellar cortex to about 1:2 in pons and medulla oblongata. In cerebral cortex, the K_m-values for monoamine oxidase A and B toward DA were found to be 240 and 230 μM, respectively (O'Carroll et al. 1983). Therefore, provided the inhibition of monoamine oxidase contributes to the postulated protective effect of cigarette smoke, the high concentration of the two βCs in tobacco smoke with differing affinity to both isoforms would make norharman and harman attractive candidates for the compounds involved. By comparing the K_i-values for norharman and harman (both βCs are not substrates of the enzyme) with the K_m-values for DA it is noteworthy that the affinity of norharman was about 330- and of harman 1,000-times higher than that of DA to the respective isoenzymes.

In conclusion, recent studies with higher concentrations of clinically used MAO B inhibitors do not support the notion of their neuroprotective actions by direct inhibition of the enzyme or by inhibition of MAO A. However, the findings do not refute the rationale behind the studies with those drugs. Dopamine is metabolized preferentially by MAO A at least in rats (Green et al., 1977). Total MAO activity is present in the brain in large amounts and it is only when both isoenzymes are inhibited the dopamine level increase. In addition, the amount of MAO B is increased in PD due to gliosis and a compensatory mechanism for the inhibition of the enzyme in smokers (Launay et al. 2009). Therefore, both isoenzymes should be inhibited to a certain extent to achieve neuroprotection by decreasing e.g. the detrimental ROS production by the enzyme activity of MAO. The correlation between the levels of the bCs norharman (inhibitor of MAO B) and harman (inhibitor of MAO A) in tobacco smoke and the proportion of the inhibited isoenzymes in the human brain strongly support the view that these two βCs are important substances responsible for the inhibition of MAO A and B in human brain and other organs. On the other hand, the large study showing smoking subjects to have a reduced risk for developing PD support the view of preferential neuroprotection of dopamine neurons by inhibition of both isoforms of MAO. The preference for dopamine neurons was suggested by the observation that the risk to develop AD was not reduced by smoking.

Although it is beyond the scope of this chapter, one additional aspect that should be mentioned concerns the contribution of βC-induced dopamine release in the ventral striatum, a brain region linked to improved mood and pleasure (e.g. Brody et al. 2008) to addiction in smokers. In vivo microdialysis experiments in rats revealed that norharman (2.44 micromole/ kg body weight, intraperitoneal application, this are

0.44 mg/ kg) and harman (2.27 micromole/ kg) caused an increased dopamine release (+70% and +72% respectively over basal) in the nucleus accumbens of rats (Sällström et al. 1995, 1996). Harman (2mg/ kg i.v.) increased the firing rate of ventral tegmental dopaminergic neurons recorded in vivo in rats. The effect was 18-times greater than that of nicotine (11 μg/ kg i.v.). The effect was not caused by inhibition of MAO A because it was not observed in rats treated with an MAO A inhibitor (befloxatone, Arib et al. 2010). Provided smoking a full cigarette results in more than 88% nicotinic receptor occupancy associated with desensitization of these receptors (Brody et al. 2006), we postulate that the βCs contribute substantially to the addictive effects of smoking, specifically to improve mood and pleasure.

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Chapter 8
β-Carbolines Increase the Performance of the Respiratory Chain in Mitochondria

Hans Rommelspacher, Monika Frenzel, and Norbert A. Dencher

Contents

Abstract The neurotoxin MPP^+ was infused into the left lateral ventricle of rat brain for 4 weeks followed by infusion of saline and 9-methyl-βC, respectively, for 2 weeks. A dose of MPP^+ was selected which reduced the level of dopamine (DA) in the striatum by approximately 50% at the end of the 6-week infusion period with MPP^+/saline. This condition should correspond to an early stage of Parkinson's disease. The mitochondrial proteome was investigated with emphasis on the composition, abundance, structure, and activity of membrane proteins and supercomplexes. We did not find changes in the catalytic activity of supercomplexes containing complex I in striatal homogenates from rats treated with MPP^+/saline. The in-gel measurement of the nicotinamide adenine dinucleotide dehydrogenase activity revealed that 9-methyl-βC stimulated the enzyme activity of complex I in rats pretreated with MPP^+ (+80%). This increase was primarily caused by a specific supercomplex ($I_1III_2IV_2$), which was approximately three times more active in MPP^+/9-methyl-βC

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than in MPP^+/saline treated rats. The abundance of complex IV was not different among groups. These findings suggest that 9-methyl-βC specifically interacts with the dimer of complex IV in supercomplex $I_1III_2IV_2$. The improvement of the performance of the respiratory chain probably contributed to the observed restorative effects of the βC by providing more ATP and by protecting mitochondria from the deleterious action of toxic oxygen species by reducing their production.

Keywords *N*-methyl-4-phenylpyridinium • Parkinson's disease • 9-Methyl-β-carboline • Dopamine • Respiratory chain • Complexes • Supercomplexes • NADH:ubiquinone reductase • Neuroprotection • ATP • Reactive oxygen species

8.1 Introduction

β-Carbolines (βCs) methylated on nitrogen in the 2-position are permanent cations and structural analogues of the neurotoxic *N*-methyl-4-phenylpyridinium ion (MPP^+). The latter compound exerts neurotoxic effects by inhibition of complex I of the respiratory chain in mitochondria (Nicklas et al. 1985). Certain N-methylated derivatives of the βCs inhibit the respiratory chain in mitochondria also, in particular complex I (Albores et al. 1990), which causes neurodegeneration (Lorenc-Koci et al. 2006). There are numerous examples of compounds that exert dose-dependent effects, which may even oppose each other related to variation of substituents e.g., behavioral activation vs. sedation by benzodiazepines (Rommelspacher et al. 1982; Pinna et al. 2006) and neuroprotection vs. neurotoxicity by ethanol (Collins et al. 2010). Thus, with respect to the βCs, they might not only inhibit but also positively modulate the respiratory chain again dependent on the substituents. In the latter case, the βCs would likely increase the efficiency of electron/proton flow and hence ATP synthesis while also minimizing the generation of potentially toxic reactive oxygen species (ROS) proposed to be involved in the pathogenesis of neurodegenerative diseases and in aging. A small degree of inactivation of complex I resulted in a significant increase in ROS formation (Sipos et al. 2003) while the arrangement of complexes to supercomplexes in the respiratory chain reduced the generation of ROS (Schägger and Pfeiffer 2000). The present view of the nature of the organization of the complexes is that the components are assembled into huge supramolecular energy-converting machines, i.e., supercomplexes with variable composition, depending, e.g., on metabolism and age (Fig. 8.1, Frenzel et al. 2010). The enzymatic activity of the individual complexes within the respective supercomplex has been determined which allowed comparison and assessment of the favorable compositions related to overall supercomplex function. Quantification of NADH dehydrogenase activity of complex I revealed that complex I in supercomplex $I_1III_2IV_1$ displayed the 2.3-fold activity of supercomplex I_1III_2. Complex III (ubiquinol-cytochrome c oxidoreductase) was active in supercomplex $I_1III_2IV_1$, but showed only minor cytochrome c reductase activity in supercomplex I_1III_2 (16.5-fold difference, Schäfer et al. 2006; complex III occurs in bovine mitochondria only as dimer). Complex IV is therefore essential for the increased activity of complexes I and III_2.

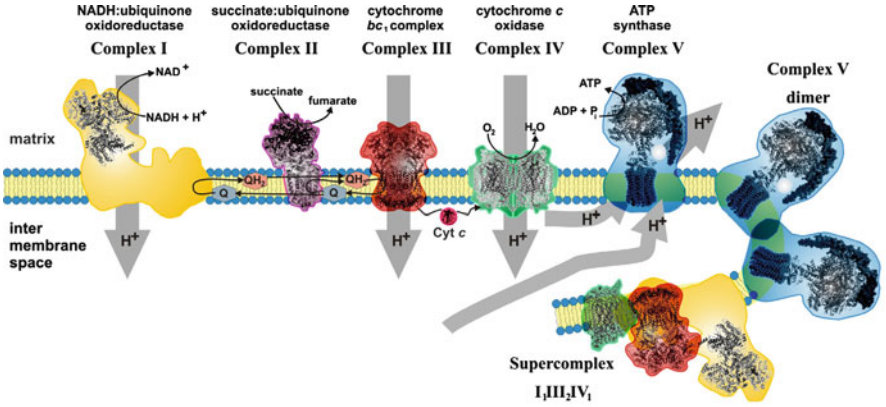

Fig. 8.1 Schematic representation of the complexes of the respiratory chain in the inner mitochondrial membrane. Electrons are transferred from NADH or succinate to the terminal acceptor molecular oxygen. Key components of this process are large transmembrane protein complexes (complexes I–IV) and the smaller mobile electron carriers ubiquinone and cytochrome c. Complexes I, III, and IV generate thereby an electrochemical proton gradient across the membrane (symbolized by *gray arrows*). The ATP synthase utilizes this gradient to produce the energy currency ATP. The representation of complex I bases on the shape from a single particle analysis of the *Yarrowia lipolytica* complex intuitively combined with the X-ray structure of the soluble domain from *Thermus thermophilus*. The illustration of complex II includes the crystal structures from the porcine heart enzyme. For the complexes III and IV the homodimeric forms from bovine heart mitochondria are shown. In case of the ATP synthase X-ray data from F_1 portion together with the c-ring and from the peripheral stalk were fitted in the contour from single particle analysis of the complete bovine heart mitochondrial complex V (from Seelert H et al (2009) Biochim Biophys Acta 1787:657–671 with permission)

The 3D map of complex $I_1III_2IV_1$ showed spatial arrangement of both complexes III_2 and IV with I. Besides higher structural stability of the complexes, these arrangements result in short diffusion distances for the small mobile electron carriers ubiquinone (also called coenzyme Q) and cytochrome c, thus facilitating the electron transfer from complex I via complex III, performed by ubiquinone, and to complex IV, performed by cytochrome c. In addition, this possibly reduces ROS generation (Seelert et al. 2009). The MF_0F_1 ATP synthase exists as a monomer, dimer, and homooligomer, probably as "ATP synthasome," composed of ATP synthase with carriers for phosphate and ADP/ATP (complex V; Schon and Dencher 2009). The ATP synthase is probably not part of a supercomplex with complexes I, III_2, and IV.

8.2 Age-Related Changes of the Respiratory Chain

The respiratory chain residing in the inner mitochondrial membrane is subject to age-associated changes in its composition, architecture, and activity (Frenzel et al. 2010). The abundance of ATP synthase was decreased 1.2-fold (even 1.5-fold in the

case of the monomer) in rat cerebral cortex of 30-month-old male rats compared to 5-month-old rats. Thus, the declined amount of ATP synthase may be related to the reduced ATP levels reported in senescent human cells (Stöckl et al. 2007, Wang et al. 2003, Zwerschke et al. 2003). Aging is accompanied by a 2.8-fold increase of unbound F_1 of complex V (containing the three ATP generating catalytic sites in the intact enzyme) which will deleteriously hydrolyse ATP in the tissue. Therefore, even a small decrease in the amount of ATP synthase might have a significant impact on ATP level. In addition, an age-associated increase in homooligomeric states (V_3–V_4) occurs at the expense of the monomeric MF_0F_1 ATP synthase. The synthase dimers and oligomers are involved in cristae formation, e.g., by inducing curvature of the inner mitochondrial membrane. The extent of cristae invagination has an effect on the spatial distribution of respiratory complexes and the efficiency of energy conversion in mitochondria (Zick et al. 2009). The extent of cristae remodeling by the observed age-associated decreased abundance of ATP synthase and increased oligomerization is not clear.

Only a small 1.2-fold age-associated decrease in the abundance of individual complexes III_2 and IV occurred. Complex I was present <5% as an individual complex in rat brain of this preparation. However, since quantification of changes in the abundance of supercomplexes revealed a significant loss of complex I containing supercomplexes (1.6-fold), overall complex I abundance declined 1.6-fold. The supercomplex I_1III_2 even declined by in amount 2.4-fold. However, the change in the relative proportion of the supercomplex $I_1III_2IV_1$ having the highest specific activity of I compared to all other supercomplexes was only 1.4-fold. This could in part compensate for the overall abundance decline in respiratory complexes and supercomplexes with age (Frenzel et al. 2010). These findings demonstrated the dynamics of the inner mitochondrial membrane supercomplexes, their enzymatic possibility to compensate for deficits, and suggested possible targets for compensating compounds.

8.3 9-Methyl-βC Increases the Performance of the Respiratory Chain In Vivo

To mimic a condition of deteriorated respiratory chain in vivo, we infused MPP^+ (dissolved in physiological saline) into the left lateral ventricle of the brain from 3-month-old rats for 4 weeks. After replacement of the osmotic mini pump, the infusion was continued by either saline or 9-methyl-βC (dissolved in saline) for 2 weeks. A dose of MPP^+ was selected which reduced the levels of dopamine (DA) in the striatum by approximately 50% at the end of the 6-week infusion period with MPP^+/saline. This condition should correspond to an early stage of Parkinson's disease (Wernicke et al. 2010). Notably, the levels of DA normalized to the amount of sham-operated controls in rats infused with MPP^+/9-methyl-βC (for details see Chap. 10). The mitochondria were isolated separately from left and right striatal tissues. The mitochondrial proteome was investigated with emphasis on the composition, abundance, structure, and activity of membrane proteins as well as supercomplexes.

The changes induced by the two treatment conditions were compared to mitochondria from rats sham operated twice. Using blue-native electrophoresis, i.e., separating proteins in their native state and preserving all functional relevant protein–protein interactions, we were able to identify and quantify distinct protein bands containing either the individual respiratory complexes or their supercomplexes with a defined stoichiometry. The complexes and the supercomplexes could already be detected in the first-dimension native gel. Unambiguous complex assignment was possible from the analysis of the subunit pattern of denatured complexes and supercomplexes in the second dimension. We did not find pronounced differences in the relative proportion of individual complexes and specific supercomplexes or in the monomeric vs. oligomeric (dimers, trimers, and tetramers) arrangements of the proton-ATP synthase. This is in line with a previous study that analyzed the substantiae nigrae, tegmentae, and cerebellae of PD patients (Schägger 1995). Others reported that in mitochondria from frontal cortex of PD patients the 8 kDa subunit of complex I was decreased in abundance by 34% and that the proteins comprising the catalytically active core of complex I were oxidatively damaged (Keeney et al. 2006). Moreover, we found that the abundance of mitochondrial HSP60, a stress response protein that acts as an important chaperone, was not affected by the various treatments (Wernicke et al. 2010).

The NADH dehydrogenase activity of complex I as an individual complex and in all supercomplexes containing complex I was similar in all striata except in the striatum from rats treated with MPP^+/9-methyl-βC. Thus, MPP^+ had been eliminated during the 2-week saline treatment period. The activity of complex I from the MPP^+/9-methyl-βC treated striata was approximately 80% higher than that from sham-operated rats and 75% higher than in rats treated with MPP^+/saline. It is worth mentioning that about 90% of all complex I was found as part of the various supercomplexes in this preparation. Further analyses of the specific NADH dehydrogenase activity of supercomplexes specific in composition revealed that only the activity of supercomplex $I_1III_2IV_2$ was approximately three times higher in the left striatum from rats with the MPP^+/9-Me-βC treatment as compared to the MPP^+/saline treatment and two times higher than sham-operated rats. The complex I activity in all other supercomplexes was unchanged. When evaluating these findings it should be taken into account that MPP^+ solely affects DA neurons which only comprise 1–2% of the population of neurons in the striatum while the effects of 9-methyl-βC probably occur in all mitochondria of both neurons and glial cells. Therefore, if residual effects of MPP^+ existed in mitochondria from DA neurons despite a washout period of 2 weeks during the infusion of saline and 9-methyl-βC respectively, we should be unable to detect them. Finally, these findings of an increased performance of the mitochondrial respiratory chain in vivo are consistent with results of a previous study. Exposition of primary embryonic murine DA neurons to 9-methyl-βC for 48 h induced an increase in ATP levels, strongly suggesting an increased performance of the respiratory chain under these in vitro conditions (Hamann et al. 2008). Besides 9-methyl-βC only methylene blue (3,7-bis-dimethylamino-phenazathionium) has been reported to exert similar protective effects on the respiratory chain (for review see Atamna and Kumar 2010).

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Chapter 9
Antioxidant Properties of β-Carbolines

Jochen Lehmann

Contents

Abstract In general all β-carbolines can be considered as molecules with antioxidant properties. On the one hand chemically, for radical consuming reasons, because of the reactivity of the indole part towards oxygen derived radicals, on the other hand enzymatically, because of monoamine oxidase inhibition. Hydrogenation of the pyridine part to tetrahydro- and dihydro-β-carbolines, as well as hydroxylation to phenolic derivatives will usually increase the chemically directed antioxidant capacity. Some studies conclude that the antioxidant activity of β-carbolines might yield into cell- and tissue protective actions including neuroprotection.

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Keywords Antioxidant • Radical • Monoamine oxidase • Tetrahydro-β-carboline • Dihydro-β-carboline • Oxidative stress • Neuroprotection • Garlic • Fruit • Fruit juice • Human eye • Reperfusion • Thrombosis

9.1 Introduction

Supply of oxygen is certainly necessary for all aerobic organisms, on the other side, oxygen can be considered as a toxic molecule. It is a diradical itself (•O–O•) and leads to formation of further oxygen derived radicals such as hydroxyl (•OH), the hyperoxide or formerly called superoxide anion ($O_2^{\bullet -}$), perhydroxyl (HOO•), or reactive molecules such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and peroxynitrite (ONO_2^-). Radicals are molecules or atoms with an unpaired single electron und thus chemically highly reactive towards not only other radicals but basically towards all endogenous molecules crossing their way. The oxygen derived radicals perform oxidation of other biomolecules which sometimes leads to their oxidative degradation, which can be concluded as oxidative stress. Oxidative degradation of unsaturated fatty acids, due to their high reactivity towards radicals at the allylic carbons in neighborhood to the double bonds, is a typical and pathophysiologically important example. The brain contains large amounts of polyunsaturated fatty acids which are accordingly highly susceptible to degradation by oxygen derived radicals and unfortunately, brain has a low antioxidant capacity.

Humans have an O_2 consumption of ~250 mL/min and approximately 2–4% of it is converted to other reactive oxygen species (ROS) (Pless et al. 1999). The highest toxicity may be attributed to the hydroxyl radicals. Those can be generated in vivo via Fenton reaction from the hydrogen peroxide which is formed endogenously in the brain (Sinet et al. 1980). And, of course, hydroxyl causes degenerative reactions such as lipid peroxidation in cell membranes. Other ROS may do the same, since in general, neurons are highly sensitive to free radical injury, have low activities of antioxidant enzyme systems, and low concentrations of free radical scavengers (Pless et al. 1999). Accordingly, the progression of neurodegenerative diseases, such as Alzheimer's disease (Papolla et al. 1997), Parkinson's disease (Götz et al. 1994), and the mental deterioration associated with Down's syndrome (Kedziora and Bartosz 1988) was shown to involve damage by free radicals.

Accordingly, an antioxidant treatment suggests itself and well-established antioxidant substances such as ascorbic acid (vitamin C) or vitamin E are recommended and in use. In the following it will be outlined by discussing results from the more recent literature that beyond these vitamins, also β-carboline derivatives, obtained by chemical synthesis or isolated from natural sources, display antioxidant properties, although they have been earlier in discussion to display phototoxic properties by producing ROS (Larson et al. 1988; Pari et al. 2000).

9.2 Antioxidant β-Carbolines

9.2.1 Indoles Versus β-Carbolines: What Is the Difference?

The radical scavenging antioxidant properties of melatonin, which is an N-acetylated 5-methoxy-indole, have been in focus up to now for many years. Lately the investigations of Herraiz and Galisteo (2004) demonstrated that not only melatonin but rather indoles in general, including several tryptamine and tryptophan derivatives, are antioxidants due to their ability to scavenge radicalic cations and then themselves turn to indolic oxidized degradation products via indolyl radicals (Fig. 9.1). Since the β-carbolines can considered as 2,3-disubstituted indoles and furthermore, the 1,2,3,4-tetrahydro-β-carbolines as cyclized tryptamines, it is no surprise that they have antioxidant capacity as well.

Theoretically, the 1,2,3,4-tetrahydro-β-carbolines should be expected to be superior to both indoles and the fully aromatic β-carbolines regarding the antioxidant

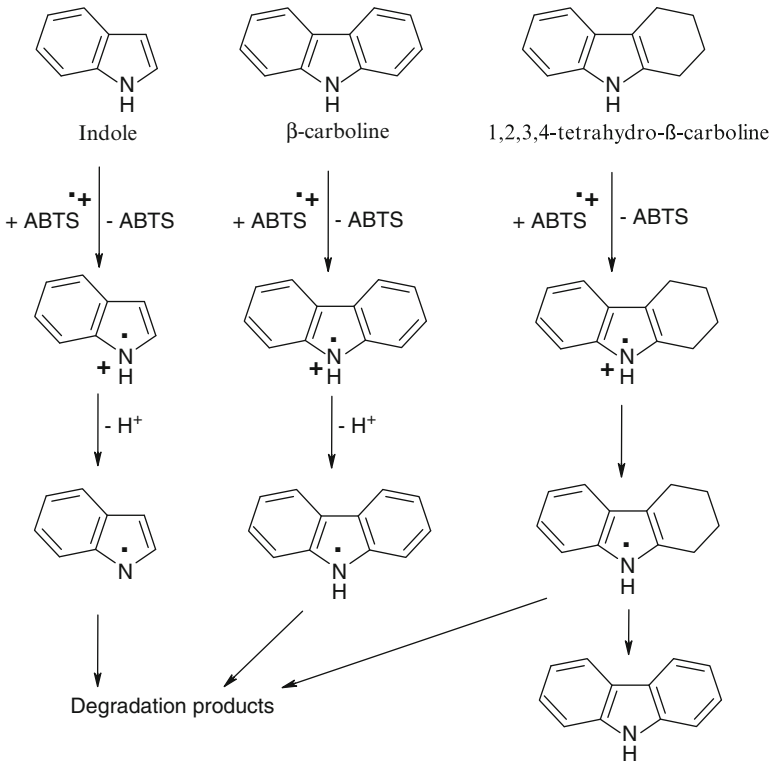
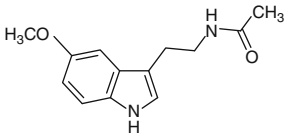
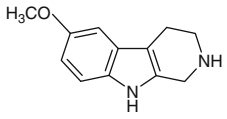


Fig. 9.1 Proposed reactivity of indoles (Herraiz and Galisteo 2004) towards ROS compared to β-carboline and 1,2,3,4-tetrahydro-β-carboline. ABTS radical cation (generated from ABTS = 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) used in the assay as model compound for ROS

Table 9.1 IC_{50} (mM) values for melatonin and pinoline in inhibiting H_2O_2-induced lipid peroxidation in brain homogenates



Melatonin



Pinoline

Cerebellum	0.66	0.13
Hypothalamus	0.51	0.13
Cortex	0.50	0.10
Hippocampus	0.35	0.06
Striatum	0.16	0.04

The IC_{50} values represent the observed mean of 50% inhibition of lipid peroxidation in the different brain homogenates. Lipid peroxidation was induced with 5 mM H_2O_2 for 60 min (taken from Pless et al. 1999)

potency, because they may consume more of the ROS by undergoing an additional oxidation from the tetrahydro- to the fully aromatic derivatives (Fig. 9.1).

Pless et al. (1999) demonstrated the advantages of tetrahydro-β-carbolines compared to indoles experimentally by measuring the antioxidant potency of melatonin and pinoline, which is 6-methoxy-1,2,3,4-tetrahydro-β-carboline, towards the commonly used oxidative model compound 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS). Furthermore, they measured the decrease in lipid peroxidation induced by these compounds. These results are given in Table 9.1 and show the activity of both compounds with the tetrahydro-β-carboline being superior to the indole derivative.

These results are contrary to the ones of Pähkla et al. (1998) who found melatonin to be more active than pinoline. But they have used a cell-free system, while the results of Pless et al. (1999) were obtained from homogenized tissues. And they are more in coincidence with the fact that a tetrahydro-β-carboline such as pinoline, contrary to a simple indole, can be oxidized further to an aromatic β-carboline and consequently should have a higher consumption of oxidizing agents. Tse et al. also confirmed that β-carbolines with a partially hydrogenated pyridine ring such as the usual tetrahydro-β-carbolines are superior with regard to antioxidant capacity compared to the fully dehydrogenated derivatives (Tse et al. 1991). In another previous study, Kawashima et al. (1995) had found the antioxidant capacity of 12 β-carboline derivatives which were structurally very similar to pinoline.

In addition to those antioxidant properties which are purely based on the chemistry of the molecules (see Fig. 9.1), another antioxidant effect specifically for the β-carbolines should be discussed: The action of monoamine oxidases may also lead to oxidative stress due to the production of hydrogen peroxide (Edmondson et al. 2009). Since many of the β-carbolines inhibit monoamine oxidases (Chap. 7) they display a second very different mode of antioxidant action, contrary to indoles in general and contrary to other antioxidants like ascorbic acid or vitamin E. Thus, β-carbolines can be considered as synergistic antioxidants with a dual mechanism.

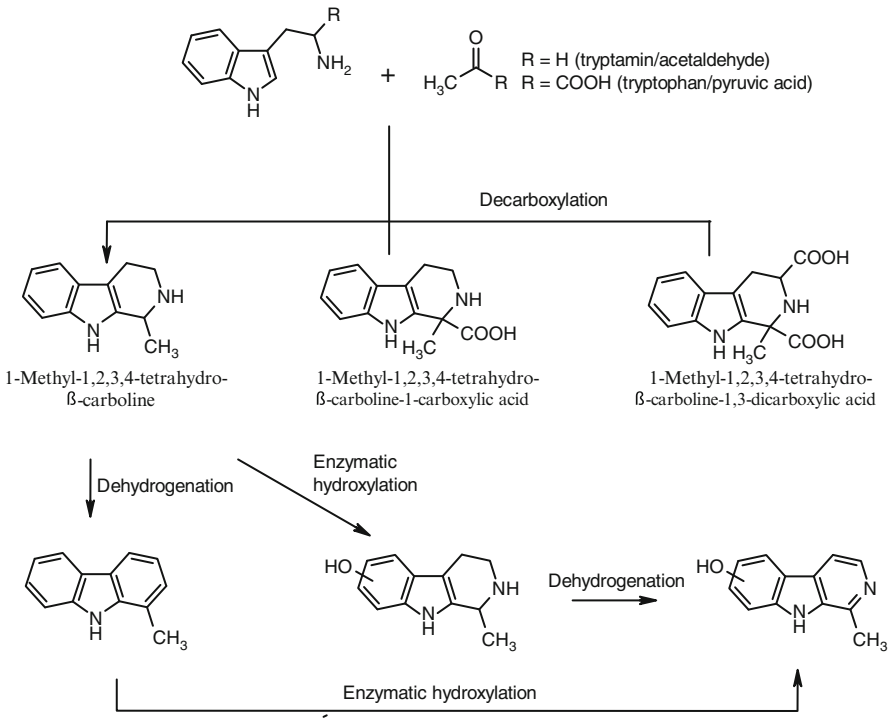
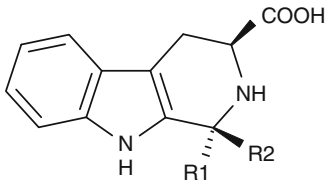


Fig. 9.2 Broad spectrum of more or less antioxidant β-carboline derivatives formed by biosynthesis and further biotransformations (Susilo and Rommelspacher 1988)

9.3 Antioxidant β-Carbolines from Natural Sources

Tetrahydro-β-carbolines are formed via Pictet–Spengler cyclization reaction between indolylethylamines, e.g., tryptamine, tryptophan (yielding β-carboline-3-carboxylic acids) and carbonyl compounds, e.g., acetaldehyde (yielding 1-methyl-tetrahydro-β-carbolines), or pyruvic acid (leading to 1-methyl-tetrahydro-β-carboline-1-carboxylic acids, see Fig. 9.2). This synthesis is a "biomimetic" one (Susilo et al. 1987). It proceeds endogenously in mammals (Susilo and Rommelspacher 1988), plants, or in the extracts taken from those during extracting or storage, without any enzymatic catalysis, just at acidic or physiological pH but also can be performed similarly in a chemical laboratory. It is quite understandable that the Pictet–Spengler reaction and other chemical transformations extending the variety of β-carboline derivatives, at least the nonenzymatic ones, also proceed during food production, fermentation, processing, or even storage. Synthesis of tryptophan- or pyruvic acid-derived β-carbolines can be followed by decarboxylation (Gynther et al. 1986; Susilo and Rommelspacher 1988), thus producing the same derivatives which result from the reaction of tryptamine or acetaldehyde. Furthermore, in enzymatically active biological systems, the primary tetrahydro-β-carbolines may metabolize via ring hydroxylation (Beck et al. 1988) to yield phenolic tetrahydro-β-carbolines, which is supposed to increase, and dehydrogenation (=oxidation=aromatization) to decrease



	R_1	R_2
(1R,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**1R3S-MTCC**)	CH_3	H
(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**1R3S-MTCC**)	CH_3	H
(1R,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (**1R3S-MTCdiC**)	CH_3	COOH
(1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (**1S,3S-MTCdiC**)	COOH	CH_3

Fig. 9.3 Tetrahydro-β-carbolines found in aged garlic extract (Ichikawa et al. 2006; Sato et al. 2006)

the antioxidant capacity (see Sect. 9.1). Methylations and other biotransformation reactions may further extend the spectrum of β-carboline derivatives with more or less antioxidant activities, available from biological tissues.

Thus, it is quite understandable that many β-carboline derivatives were originally isolated from biological materials and also total extracts of plants or other tissues have been investigated on their antioxidant capacity.

9.3.1 Garlic

Garlic (*Allium sativum*) has been used not only for flavoring food but also as functional food and in traditional medicine. Antioxidant properties have been reported already before the β-carboline discussion came up (Rietz et al. 1993; Numagami et al. 1996; Ide et al. 1996). The organic sulfur compounds in garlic have been postulated to be responsible for beneficial properties such as antioxidant activity (Imai et al. 1994; Ide and Lau 1997, 1999), cancer prevention (Amagase and Milner 1993), and antiatherogenic (Efendy et al. 1997) and antiplatelet aggregation activity (Rahman and Billington 2000; Steiner and Li 2001). But in fact, garlic contains a variety of β-carbolines, especially the aged garlic extract (AGE). AGE is manufactured by a long-term extracting process, which takes more than 10 months at room temperature. Since biosynthesis and biotransformation of β-carbolines is expected to go on during extraction time, the variety and the amount of β-carbolines in AGE (Ichikawa et al. 2006) but also in is short-term fermented (40 days at 60–70°C) garlic (Sato et al. 2006) is superior to that in raw, sliced, baked, boiled, crushed, or freeze-dried garlic preparations (Ichikawa et al. 2006; Sato et al. 2006).

Both 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids and the corresponding 1,3-dicarboxylic acids have two chiral carbons and four stereoisomers are possible of each. In total, only four of those eight (Fig. 9.3) were found even in the AGE (Ichikawa et al. 2006). The *S*-tryptophan derived part of the molecule keeps its *S*-configuration in the corresponding tetrahydro-β-carboline-3-carboxylic acids

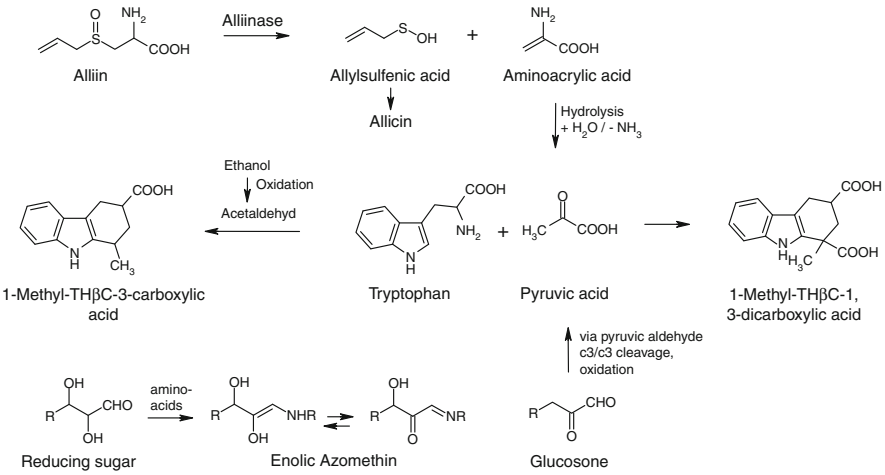


Fig. 9.4 Organic sulfur compounds, sugars in garlic, and alcohol may enhance the synthesis of tetrahydro-β-carboline-carboxylic and -dicarboxylic acids (taken from Ichikawa et al. 2006)

(position 3), otherwise Fig. 9.3 would show eight instead of four compounds. Obviously, stereochemistry is relevant regarding the antioxidant capacity. Ichikawa et al. found 1*S*,3*S*-MTCdiC easily reaching the potency of ascorbic acid and superior to the other three compounds in Fig. 9.3 (Ichikawa et al. 2006).

Conclusively, these authors (Ichikawa et al. 2006) pointed out that there is a very special situation in garlic since the typical organosulfur compounds contribute to the formation of β-carboline-carboxylic acids as outlined in Fig. 9.4 for the cyclization of tryptophan. Any processing of garlic, even just cutting it, causes the major organosulfur compound alliin to be transformed into allicin via allylsulfenic acid. Pyruvic acid, which is able to generate tetrahydro-β-carboline-1-carboxylic acids is formed as a byproduct in this reaction. Further pyruvic acid can be formed by oxidation of the pyruvic aldehyde which results from reducing sugars by the Maillard reaction pathway (Wnorowski and Yaylayan 2000). Last but not least, ethanol is easily oxidized to acetaldehyde, which is highly reactive in the Pictet–Spengler cyclization. It is obvious that 1-methyl-β-carboline synthesis can be promoted whenever ethanol is present in any processing or extraction of garlic (AGE!) or other biological materials containing tryptophan or tryptamine.

After all, extracts of garlic contain two different synergistic antioxidant principles, both the sulfur compounds and the β-carbolines and furthermore the presence of ethanol creates a chemical setting which notably enhances the formation of β-carboline derivatives.

9.3.2 Fruits and Fruit Juices

It is understandable that all biological materials which contain amino acids or proteins, serving as a source of tryptophan or tryptamine, and carbohydrates, as a source

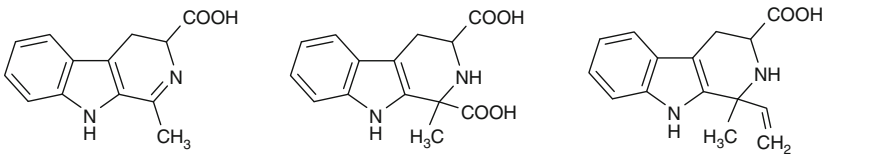
of pyruvate or acetaldehyde, are expected to accumulate β-carbolines. Accordingly, Herraiz and Galisteo found 0.02–0.66 mg/L 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (TCC) and the steroisomeric 1*S*,3*S*-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*S*,3*S*-MTCC) and 1*R*,3*S*-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1*R*,3*S*-MTCC) in all of the investigated juices tomato, peach, pear, apple, orange, grapefruit, kiwi, pineapple, banana, tropical fruits, and grape. Also, up to 1.69 mg/L in banana and 2.03 mg/L in tomato of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (HMTC) was found in several of those fruits, but not in peach, pear, apple, orange, grapefruit, and grape. 1-Methyl-1,2,3,4-tetrahydro-β-carboline (MTC) was only found in kiwi (0.39 mg/L) and tomato (0.13 mg/L), but not or very little in all the other fruit juices (Herraiz and Galisteo 2003).

Antioxidant potency was measured for 1*R*,3*S*-MTCC, MTC, TCC, and HMTC by radical scavenging of the cationic ABTS radical and all of these compounds, above all the phenolic HMTC, proved to be superior to both ascorbic acid and Trolox, which is the soluble form of vitamin E. Again, fruit juices distinguish themselves by two different antioxidant ingredients: ascorbic acid and the β-carbolines. Based on the in vitro results of Herraiz and Galisteo (2003) and assuming that absorption and bioavailability of the β-carbolines are much better than of the highly hydrophilic ascorbic acid, it can be concluded that the β-carbolines represent the more relevant one.

9.4 Protective Effects of β-Carbolines

As outlined above, several in vitro models revealed antioxidant properties of β-carbolines. Further studies suggest that these activities turn into general protective effects against oxidative stress. After Tse et al. had demonstrated the antioxidant effects of β-carbolines by measuring thiobarbiturate reactive products formed by lipid oxidation (Tse et al. 1991), Garcia et al. went further and showed a brain-protective effect of the very simple 1,2,3,4-tetrahydro-β-carboline (tryptoline) and its 6-methoxy analogue pinoline in homogenated rat brain. Lipid peroxidation induced by hydrogen peroxide was totally prevented and based on these results the authors speculate that these β-carbolines may be neuroprotective agents (Garcia et al. 2000).

Lee et al. used not only PC12 cells in culture but also the mouse brain in vivo to elucidate the protective effects of some β-carbolines (harmaline, harmalol, and harmin) on oxidative neuronal damage (Lee et al. 2000). They found that treatment of mice with MPTP increases the activities of superoxide dismutase, catalase, and glutathione peroxidase, and enhances the formation of tissue peroxidation products such as malondialdehyde. But a coadministration of 48 mg/kg harmalol attenuates all these MPTP effects. Harmaline, harmalol, and harmine also attenuated both the MPP+-induced inhibition of electron flow plus membrane potential formation and the dopamine-induced thiol oxidation in the mitochondria. Accordingly, a dopamine-induced viability loss in PC12 cells was also attenuated by these β-carbolines. A scavenging action of hydroxyl radicals was confirmed by the inhibition of deoxyribose degradation, the β-carbolines alone did not exhibit any cytotoxic effects. The authors conclude that both



1-Methyl-3,4-dihydro-β-carboline-3-carboxylic acid (**MDCC**)

1-Methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (**MTCDC**)

1-Methyl-1-vinyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**MVTCC**)

Fig. 9.5 β-Carbolines found in the human eye lens (Pari et al. 2000)

dopamine-induced brain mitochondrial damage in vivo and PC12 cell death in culture are prevented through a scavenging action on ROS, the blocking monoamine oxidase and thiol oxidation. In a following multitarget in vitro study, including the rate of vitamin E disappearance, DPPH radical scavenging, aggregation of the LDL protein induced by oxidation, and LDL oxidation induced by copper, Berrougi et al. confirmed the antioxidant and potentially protective properties of β-carbolines and they found harmaline to be superior to harmine (Berrougui et al. 2006).

9.4.1 Human Eye

Meanwhile, it is well known that β-carbolines are found ubiquitously in a variety of foods and, perhaps as a consequence of this, but certainly also by independent biosynthesis, appear in animal and human fluids and tissues such as blood, urine, kidney, liver, and brain. Some β-carbolines (Fig. 9.5) were even found in the lenses of the human eye (Manabe et al. 1996; Dillon et al. 1976), among them the 1-vinyl derivative MVTCC (Pari et al. 2000). The quantities of these β-carbolines increase with age and are the highest in senile cataract and diabetic cataract lenses (Pari et al. 2000). Pari et al. have shown that these lenticular β-carbolines have little or no adverse photodynamic properties but rather display an antioxidant and thus a protective role. They quench singlet oxygen, superoxide, and hydroxyl radicals. Beyond this, they inhibit the oxidative formation of higher aggregates of a relevant protein named eye lens β-crystallin. All these findings support the hypothesis that the eye lens derived β-carbolines do not induce a cataractic disease but rather act protective in the eye.

9.4.2 Reperfusion Injury and Thrombosis

Atherosclerotic plaques lead to a prolonged reduction in coronary blood flow and consequently to necrotic damages of the myocardium. On the other hand, reperfusion of a hypoxic cardiac tissue with fresh blood and oxygen will paradoxically produce further cell damage. This type of reperfusion injury is hypothesized to be associated with the generation of oxygen derived radicals after resupply with relatively excessive oxygen.

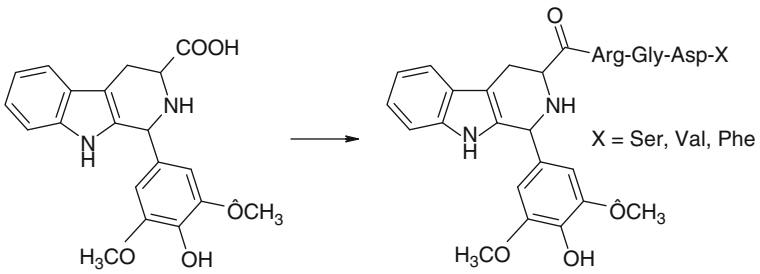


Fig. 9.6 Tetrahydro-β-carboline-RGD-peptide hybrid compounds with antioxidant and antithrombotic activity

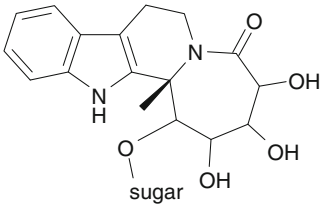


Fig. 9.7 MAO A inhibitory and antioxidant banistenoside from *Banosteriopsis caapi*

Bi et al. have synthesized hybrid compounds which combine a phenolic substituted tetrahydro-β-carboline with a short peptide side chain (Fig. 9.6) and they showed that these β-carbolines are scavengers of free radicals such as ·OH, ·NO in PC12 cells and also neutralize the action of hydrogen peroxide. Scavenging of the endogenous vasodilator NO (nitric oxide) was further confirmed by a vasorelaxation assay. Last but not least, the β-carboline-peptide derivatives showed to be efficacious in the treatment of rat arterial thrombosis and were also active in a platelet aggregation assay. Since both free radicals and thrombogenesis are important risk factors in myocardial ischemic/reperfusion injuries, the radical scavenging and antithrombotic activities of these β-carboline derivatives suggest them as an option for an appropriate treatment.

9.4.3 Neuroprotection

Banisteriopsis caapi is a tropical South American genus and its aqueous extract was found to display MAO inhibitory and antioxidant properties. Samoylenko et al. (2010) isolated and identified harmol, tetrahydroharmin, harmaline, and harmin but also novel azepino(1,2-*a*)tetrahydro-β-carboline derivatives (banistenosides, Fig. 9.7) as active components with regard to antioxidant activity. Due to both chemical antioxidant reactivity and enzymatic inhibition of human brain monoamine oxidase, resulting from these β-carbolines, the authors postulate a claim for the treatment of parkinsonism, including other neurodegenerative disorders.

9.4.4 Survival from Oxidative Stress

Another step from antioxidant effects in vitro to protective properties in vivo was performed by using *Saccharomyces cerevisiae* strains, proficient and deficient in antioxidant defense, for investigating the antioxidant β-carbolines harmane, harmine, harmol, and the dihydro-β-carbolines harmaline and harmalol in vivo. A significant survival of yeast cells exposed to H_2O_2 or paraquat was observed in case of pretreatment with one of the β-carboline derivatives mentioned above, as a consequence of their antioxidant effect. Again, the phenolic 7-hydroxy-dihydro-β-carboline harmalol demonstrated the strongest antioxidant effect with regard to the survival time of yeast cells (Moura et al. 2007).

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Chapter 10
Restoration of Damaged Dopamine Neurons

Hans Rommelspacher and Catrin Wernicke

Contents

Abstract Several in vitro and in vivo studies demonstrate that some β-carbolines (βC) exert neuroprotective effects. The noncationic 9-methyl-βC enhanced the enzyme activity of triosephosphate isomerase (TPI), whereas the cationic 2-methyl-βC and 2,9-dimethyl-βC inhibited enzyme activity. TPI is a rate-limiting enzyme for glycolysis and its activation improves the energy supply of the cell. In murine primary neurons, exposition to 9-methyl-βC increased the number of dopamine (DA) neurons by up to 50% and of gene transcripts of several genes involved in differentiation while transcripts of proinflammation, proapoptosis, and stress were downregulated. Chronic exposure to rotenone, a toxin which acts by inhibiting complex I of the respiratory chain, decreased the number of DA neurons. After rotenone withdrawal further deterioration was observed as well as ongoing proliferation of microglia.

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Exposure of cultures to 9-methyl-βC after rotenone withdrawal, resulted in conspicuous regeneration of DA neurons and a reversal of the number of microglia. In an animal model of PD, 9-methyl-βC normalized the decline of DA levels in the striatum. Expression of genes involved in neuronal differentiation, regeneration, and survival were upregulated including brain-derived neurotrophic factor, conserved DA neurotrophic factor, and nerve growth factor while transcripts of inflammation and apoptosis-inducing factors were downregulated. Further neuroprotective effects are reported in Chaps. 8 and 9.

Keywords Triosephosphate isomerase • 9-Methyl-ß-carboline • Murine primary neurones • Genes • Inflammation • Apoptosis • Neurotrophins • BDNF • NGF • CDNF • GDNF • Armetl • Rat • Parkinson's disease • Dopamine • Striatum

10.1 Introduction

β-carbolines (βCs) are produced in mammals from tryptophan and tryptophan-derived indolealkylamines including serotonin. Autoradiographic studies revealed enriched high-affinity binding sites of [^{3}H]norharman ([^{3}H]βC) in locus coeruleus > hypothalamus, thalamus > nucleus accumbens, amygdaloid nuclei, hippocampus > neocortex, and olfactory-related structures (Pawlik et al. 1990). Further studies demonstrated three binding sites in the forebrain of rats using a low concentration of [^{3}H]βC (~2 nM) (May et al. 1994). Pharmacological investigations identified one of them as monoamineoxidase B comprising 85% of labeled sites. [^{3}H]βC bound to the two non-MAO B sites with high affinity (86 and 560 nM resp.; about equal amount of the sites). The high-affinity binding sites in bovine medulla displayed a similar pharmacology, while those in the liver were different. The binding sites were specific for βCs because the affinity of other compounds was lower, e.g., tryptamine (230 nM), benzodiazepines (8 μM), serotonin (180 μM), muscarinic cholinergic receptor ligands (133 μM), dopamine D2 receptor ligands (317 μM), and steroids with the least affinity (Müller et al. 1981, Pawlik and Rommelspacher 1988, May et al. 1994). The two sites were postulated to represent specific βC binding sites. This is in line with findings of extracellular single-unit recording experiments in rat nucleus accumbens neurons superfused with low concentrations of harman (1-methyl-βC). Harman (10^{-11} to 10^{-9} mol/L) activated 80% of neurons while higher concentrations (10^{-8} to 10^{-6} mol/L) inhibited 75% of neurons, identified as dopamine (DA) neurons (Ergene and Schoener 1993). The high harman sensitivity of mesolimbic DA neurons was confirmed by a recent in vivo electrophysiological study utilizing intravenous drug application. Harman (2 mg/kg) increased the firing rate approximately 18 times more than nicotine (11 μg/kg), whereas the activation of DA neurons by norharman (2 mg/kg) was in the same range as nicotine (~threefold). The effect of harman was not due to MAO A inhibition (Arib et al. 2010). These and other studies using in vivo microdialysis demonstrated that norharman and harman display a preference for DA neurons with stimulation of

DA release by low doses and inhibition by higher ones, despite their serotonin-like chemical structure (Sällström Baum et al. 1995, 1996).

10.2 In Vitro Studies

It is well known that the βCs induce a broad spectrum of effects dependent on the substituents. Due to their structural homology to the neurotoxin 1-methyl-4-phenyl-pyridinium ion (MPP^+), βCs were investigated with respect to their neurotoxic properties. In several investigations it was shown that some βCs may exert neurotoxic/cytotoxic properties. However, we demonstrated that several derivatives exerted different actions. For instance, the permanent cationic derivative 2,9-dimethyl-βC induced ROS-production and apoptosis in murine Neuro2a neuroblastoma cells comparable to MPP^+. The noncationic derivative norharman did not induce ROS-production and apoptosis in the same concentration range (Pavlovic et al. 2006). In a structure-activity investigation (Wernicke et al. 2007) we demonstrated that cationic βCs are primarily transported by DAT (high-affinity DA transporter), whereas noncationic derivatives may penetrate the plasma membrane and mitochondrial membrane by diffusion. Furthermore, the noncationic βC, 9-methyl-βC enhanced the enzyme activity of triosephosphate isomerase (TPI), whereas the cationic βCs 2-methyl-βC and 2,9-dimethyl-βC inhibited enzyme activity (Bonnet et al. 2004). TPI is important for glycolysis and activation of the enzyme by this pathway improves the energy supply of the cell. We therefore rationalized that some βCs exert neuroprotective and even neurorestorative effects, whereas others are neurotoxic dependent on their substituents. To explore this possibility, we selected two cell models with dopaminergic properties because βCs showed a preference for those neurons (see Sect. 10.1). The hypothesis that 9-methyl-βC may exert neuroprotective properties was first investigated in murine primary embryonic neurons from the mesencephalon (Hamann et al. 2008). Primary cells were exposed to 9-methyl-βC for 48 h. DA neurons were identified by tyrosine hydroxylase immunostaining (THir). The number of DA neurons increased by approximately 20% in contrast to the numbers of control cells which remained constant during the short time period. Nevertheless, it was possible that a dynamic equilibrium existed between the spontaneous death of cells and induction of new neurons, including their differentiation under control conditions (Maxwell et al. 2005). DA neurons exposed to the βC produced a higher number and greater length of neurites with more ramifications than control cells. DA levels were slightly increased as was the high-affinity uptake of [^{3}H]DA. The total number of cells did not change within 48 h, while the total number of neurons tended to be increased and that of DA neurons increased significantly. This increase was not due to an enhanced proliferation of THir cells as the number of BrdU-positive cells was decreased. Lactate dehydrogenase (LDH), a measure of the demise of cells, was reduced in the medium of exposed cultures, the number of necrotic cells was diminished by 50% and caspase 3 activity was reduced suggesting cell-protective and antiapoptotic actions of the βC.

The increased level of ATP may have contributed to an improved energy supply in exposed cells due to activated glycolysis and improved efficiency of the respiratory chain (see Chap. 8). Gene transcription analyses after 24 h of exposure to 9-methyl-βC revealed increased markers of DA neurons in particular DAT, TH, and the aldehyde dehydrogenase family 1, subfamily 1 which is the only presently available marker of DA progenitor cells. The transcripts of several genes involved in DA neuron cell differentiation were increased (sonic hedgehog, promotes specification of neuroepithelial cells; Wnt1, increases proliferation and neurogenesis of ventral mesencephalic precursor cells and promotes the generation of TH^+ cells from Nurr 1-expressing precursors; and Wnt5a, which promotes the generation of TH^+ cells from Nurr1-expressing precursors more potently than Wnt1), while downregulation of transcripts of proinflammation and proapoptosis genes was observed. Notably, these experiments were conducted with embryonic tissue. Three independent regulatory cascades have been characterized in early postmitotic, maturating, and mature DA neurons (Vitalis et al. 2005). From each cascade a member was selected: engrailed1, orphan nuclear receptor Nurr1, and paired-like homeodomain transcription factor Pitx3. All three factors were increased by 9-methyl-βC. Using a PCR-array, genes involved in differentiation were upregulated (hedgehog pathway: bone morphogenic proteins Bmp2 and 4, hedgehog interacting protein (Hhip), Wnt pathway: lymphoid enhancer binding factor 1 (Lef1), vascular endothelial growth factor A (Vegfa)), whereas genes involved in inflammation (Jak-Stat pathway: chemokine ligand 9 (Cxcl9), interferon regulatory factor (Irf1), NFAT pathway: Fas ligand (Fasl; Tnf superfamily member 6), NFkB pathway: intercellular adhesion molecule1 (Icam1), tumor necrosis factor α (Tnfα), vascular cell adhesion molecule1 (Vcam1), mitosis (potassium voltage-gated channel, shaker-related subfamily beta member 2 (Nab2) and apoptosis (p53 pathway: death domain associated protein Fas, growth arrest and DNA-damage-inducible protein 45 alpha (Gadd45a), and stress (heat-shock protein1 (Hspb1, synonymous: Hsp25)) were downregulated. Additionally, glycogensynthase1 (Gys1) and hexokinase2 (Hk2), which belong to the insulin pathway, were both upregulated. Taken together, these findings indicate a pronounced shift from proliferation to differentiation and the activation of anti-apoptotic factors, as well as the reduction of the transcription of genes involved in inflammation and stress. This initial study identified 9-methyl-βC as a compound with dual actions, capable of increasing the number of functional DA neurons by upregulating DA transcription factors including specific markers and by exerting neuroprotective effects (Hamann et al. 2008).

The differentiating properties of 9-methyl-βC were investigated in the human neuroblastoma cell line SH-SY5Y. The proliferation was reduced, whereas the cells differentiated into a neuronal phenotype with long branched neurites, which created a network between the clusters of neuron-like cells.

Considering the short exposure of the culture to 9-methyl-βC it was not necessarily expected that the additional mature DA neurons were primarily derived from neuronal stem cells, progenitor, or precursor cells. An alternative explanation would be that previously THir-negative DA neurons and/or neurons of a different type resulted in DA neurons from trans-differentiation processes, a question answered in

the subsequently reported study. Again primary embryonic cultures were utilized. The increase in THir neurons exposed to 9-methyl-βC reached a maximum of 48% above controls within 48 h (Polanski et al. 2010). This effect could be explained by recruitment of preexisting DOPA decarboxylase (DDC)-positive rather than THir-negative neurons. 9-Methyl-βC induced the transcription of several TH-relevant transcription factors which may have explained these effects. In addition, cultures were exposed to EdU, a thymidine analog that is incorporated into DNA during active synthesis. Coexposure with 9-methyl-βC did not augment the incorporation of EdU indicating little recruitment of progenitor cells, if at all. When 9-methyl-βC was withdrawn, the number of THir neurons decreased to 8–10% above control values and was maintained at this level until the end of the observation period. The rapid decline of THir neurons may be attributed to the short, 68 h, half-life of the TH enzyme, reported in the adrenal medulla (Chuang et al. 1975). Chronic exposure to 9-methyl-βC maintained the 20–30% increased numbers of THir cells, while the expression of TH protein was increased by 75% indicating a higher level of enzyme per cell. The anti-inflammatory action of 9-methyl-βC was confirmed in addition to a reduction of the transcription of the respective genes. A strong reduction of the toxin-induced microglial reaction and of LDH levels in the medium was also reported. Preincubation and co-incubation of 9-methyl-βC with toxins of different modes of action prevented the proliferation of microglia and the increase in levels of LDH, in particular lipopolysaccharide (LPS), MPP^+, rotenone, and 2,9-dimethyl-βC. The toxin-induced decrease in numbers of THir cells was reversed with LPS and the βC, whereas the number was only slightly affected with rotenone and MPP^+. Co-treatment with LPS and 9-methyl-βC increased the expression of THir, DDC, Creb I, and Crebbp markedly compared with 9-methyl-βC alone. These factors are important for functionality and/or survival (Parlato et al. 2006). Chronic exposure (6 days) to rotenone, a toxin which acts by inhibiting complex I of the respiratory chain, decreased the number of THir neurons to 67% compared to control cultures. After rotenone withdrawal further deterioration of THir neurons to 54% was observed compared with controls, as well as ongoing proliferation of microglia up to 384%. After exposure to 9-methyl-βC (8 days) after rotenone withdrawal, cultures showed conspicuous regeneration of THir neurons up to 87% of control and a reversal of the number of microglia. Thus, 9-methyl-βC emerged as a compound with restorative potential for DA neurons and anti-inflammatory actions.

Recently, the antihistamine dimebon with a tetrahydro-γC structure was reported to block apoptosis of cortical neurons exposed to amyloid beta peptide (Bachurin et al. 2001). Anecdotal evidence of its efficacy against Alzheimer's disease (AD) and age-related cognitive decline (O'Brien 2008, Burns and Jacoby 2008) prompted clinical trials to determine dimebon's efficacy in treating several forms of neurodegenerative diseases. While phase II data appeared exceptionally encouraging (Doody et al. 2008), results from a phase III study showed that dimebon offered no benefit for patients suffering from AD (Miller 2010). We initiated a study to compare the efficacy of 9-monofluorethyl-γC (9-Feth-γC) and 9-trifluorethyl-γC (9-triFeth-γC) with 9-methyl-βC in the human neuroblastoma SH-SY5Y cell model.

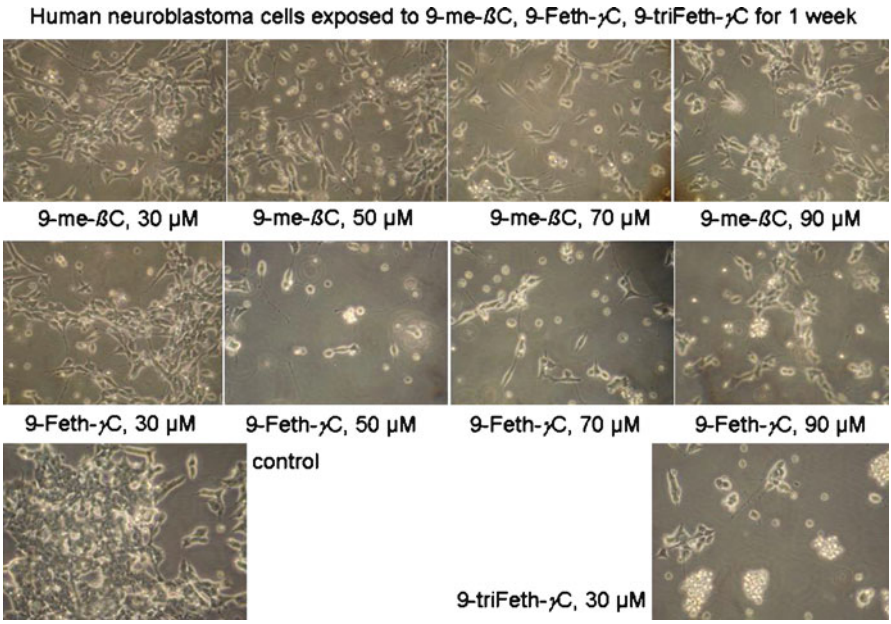

Fig. 10.1 Effect of increasing concentrations of the γ-carbolines 9-monofluorethyl-γC (9-Feth-γC) and 9-trifluorethyl-γC (9-triFeth-γC) in comparison with 9-methyl-βC on the morphology of human neuroblastoma SH-SY5Y cell after 7 days of exposure

Figure 10.1 shows the effect of increasing concentrations of the test compounds after 7 days of exposure. All three compounds reduced proliferation and switched the cells toward differentiation. The neuronal phenotype was expressed instead of the indifferentiated phenotype, with smaller and narrower cell bodies, some of them more roundish and with an increasing number and length of neurites per cell. The cultures exposed to 9-Feth-γC showed more accumulation of cell debris than 9-methyl-βC, especially at the higher concentrations. This suggested toxic actions, which were even more marked in 9-triFeth-γC exposed cultures. To investigate the toxicity of the substances, a cell viability test confirmed that the γCs were more toxic than 9-methyl-βC and that toxicity increased with an increasing number of fluoride-substituents (Fig. 10.2).

10.3 In Vivo Studies

Based upon the in vitro findings, we investigated the neuroregenerative effect of 9-methyl-βC in an animal model of PD (Wernicke et al. 2010). Rats received the neurotoxin MPP^+ via a micropump which constantly injected the neurotoxin into the left ventricle for 4 weeks. The amount was adjusted to produce a 50% reduction of

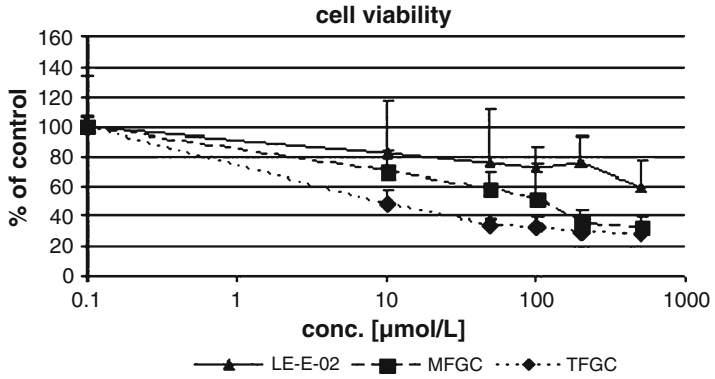

Fig. 10.2 Toxicity of the γ-carbolines 9-monofluorethyl-γC (9-Feth-γC) and 9-trifluorethyl-γC (9-triFeth-γC) in comparison with 9-methyl-βC

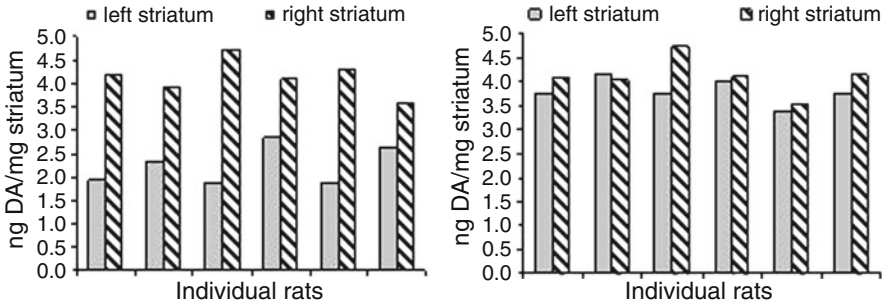

Fig. 10.3 Concentration of dopamine (DA) in the left striatum (*filled columns*) and the right striatum (*hatched columns*) of individual rats. The animals were treated with 1-methyl-4-phenylpyridinium (MPP^+) continuously for 28 days delivered into the anterior left ventricle of the brain, followed by either saline for 14 days (*left plot*) or 9-methyl-βC (*right plot*). The levels of DA were measured after the 42-day period. The DA levels in the left striata differed significantly between the MPP^+/saline and the MPP^+/9-methyl-βC treated rats ($p < 0.01$)

DA in the left striatum. The right side served as an individual control. Afterwards the pumps were replaced and the animals received either 9-methyl-βC or equimolar saline for 14 days. Rats sham-operated and vehicle-injected twice served as controls. Stereological investigation of the substantia nigra pars compacta (SNpc) revealed a significant reduction of the number and density of THir cells on the left side of MPP^+/saline-treated animals, whereas the number of THir cells and density were normalized in MPP^+/9-methyl-βC-treated animals compared to the right SNpc of the respective animal. The amount of DA was reduced up to 50% in the left striatum of MPP^+/saline-treated animals. Combined treatment rescued DA levels in the left striatum up to the amount of the right striatum of each individual animal (Fig. 10.3).

It is well established that DA neurons receive neurotrophic support from neighboring cells and vice versa in the striatum. Therefore, we investigated the expression

of 84 genes involved in neuronal cell growth, differentiation, regeneration, and survival in the striatum using real-time RT-PCR array for neurotrophins (RT Profiler PCR Array for rat neurotrophins and receptors version 2.0 by SuperArray). In order to confirm and extend the findings, we applied single real-time RT-PCR using FRET-probes for selected genes. A number of factors which are known to be important in neuronal survival were upregulated. The most obvious results were previously reported (Wernicke et al. 2010). These included the upregulation of several transcripts involved in neurotrophic support and cell survival by MPP^+/9-methyl-βC compared to sham-operated and MPP^+/saline-treated animals. Specific transcripts upregulated included brain-derived neurotrophic factor (BDNF), cerebellin1 (Cbln1), conserved dopamine neurotrophic factor (CDNF, also denoted Armetl1), nerve growth factor (Ngf), the receptors for glial-derived neurotrophic factors (GDNF), and ciliary neurotrophic factor (CNTF).

Tyrosine hydroxylase (TH) was not changed in the striatum at the transcription level, but was enhanced at the protein level. The increased TH level per cell produced by 9-methyl-βC was reported for primary neurons as well (see above). The conflicting findings concerning the transcription and protein level of TH may be based on the fact that mRNA is located in the cell bodies of DA neurons and the mature protein is located only in the nerve terminals. The heat-shock protein b1 (Hspb1) was upregulated in the right striatum and was further enhanced after combined treatment with MPP^+/9-methyl-βC on the right side and increased on the left side. Hspb1 was shown to be involved in the control of differentiation of olfactory precursor cells (Mehlen et al. 1999) and in the protection against α-synuclein-induced apoptosis (Zourlidou et al. 2004). It is downregulated in a mouse model of ALS (Maatkamp et al. 2004).

In addition to these findings (Wernicke et al. 2010), there were further differences in the transcription of several genes between the three groups of rats investigated, which were less prominent or did not reach statistical significance, mainly due to the high degree of variability. The importance of changes in transcription lies not only in the amount of change with respect to a single gene, but also in the pattern of changes in a cluster of genes with related functions.

10.4 Upregulation of Neurotrophic Factors by 9-Methyl-βC

Figure 10.4 illustrates the transcription changes for genes involved in neurotrophic support and survival presented as percent of control in the left and right striatum, respectively. Artemin (Artn) belongs to the GDNF family of neurotrophins. It binds to the GDNF family receptor alpha (Gfra) and signals through the RET receptor tyrosine kinase. Artn has been reported to be upregulated in the 6-OHDA-lesioned striatum of rats. Furthermore, lentiviral gene transfer of Artn into the striatum prevented nigral DA neurons from 6-OHDA-induced cell death (Rosenblad et al. 2000). Further, chronic administration of Artn increased survival and morphological differentiation of THir neurons (Zihlmann et al. 2005). In our experiment, Artn was

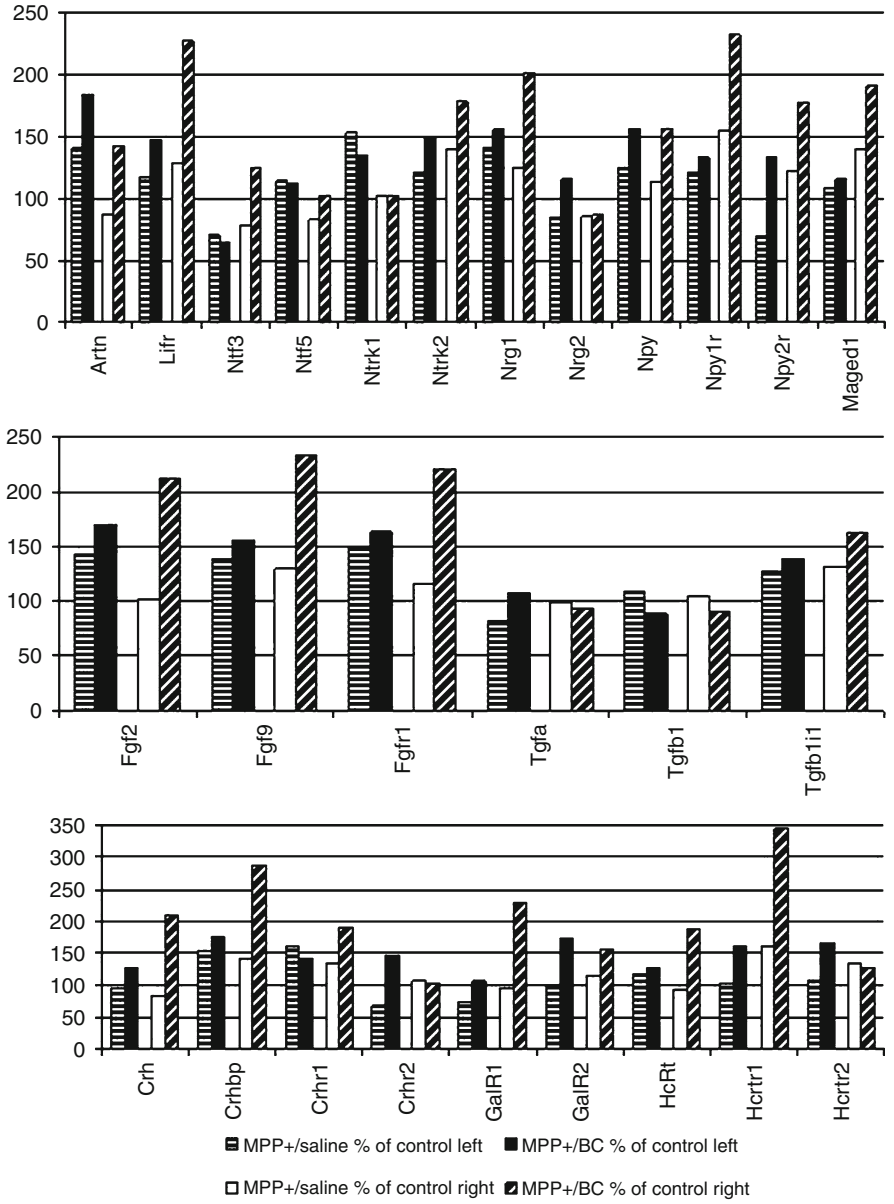

Fig. 10.4 Depicted are transcription changes of neurotrophic and related factors in the left and right striatum after MPP^+/saline and MPP^+/9-methyl-βC treatment compared to sham-operated animals

upregulated by 50% after MPP+/saline treatment on the left side and unchanged on the right side. MPP+/9-methyl-βC treatment enhanced expression by 80 and 50%, respectively. As reported earlier Gfra levels were also upregulated by MPP+/9-methyl-βC treatment in the same experiment (Wernicke et al. 2010).

Leukemia inhibitory factor (Lif) was reported to promote auditory neurons survival of early postnatal spinal ganglion cells from rats and potentiates neurotrophin-3-mediated survival synergistically (Marzella et al. 1997). The expression level of Lif was below the detection limit of this method. However the receptor was slightly enhanced after MPP+/saline treatment and further enhanced by 50 and 130% after MPP+/9-methyl-βC treatment on both sides.

Neurotrophic factor 3 (Ntf3) was downregulated by 40% on the left side by both treatments and by 20% on the right side after MPP+/saline treatment which was enhanced by 20% after MPP+/9-methyl-βC treatment. Ntf5 was nearly unaltered. Neurotrophin tyrosine kinase1 (Ntrk1) and 2, also denoted as TrkA and B, are known to mediate the differentiation and protective effects of several neurotrophic factors including NGF and BDNF. Baydyuk et al. (2011) demonstrated that a chronically reduced TrkB signaling leads to selective late-onset degeneration of DA neurons. In our setting Ntrk1 expression was only upregulated on the left side under both treatments, and Ntrk2 was upregulated by MPP+/saline by 20 and 40% on both sides, respectively. MPP+/9-methyl-βC increased the expression by 50 and 80%, respectively.

Neuregulins (Nrg) are polypeptide factors that influence growth and differentiation through interaction with the Erbb family of receptors. Erbb4 is expressed in midbrain DA neurons and was significantly reduced in the SNpc but not in the VTA in middle-aged and old rats compared to young rats. This decrease was accompanied by a decreased TH expression in the SN (Dickerson et al. 2009). These findings implicate a role for Nrg/Erbb4 in the trophic support of DA neurons in the SN. In the cerebral spinal fluid the levels of neuregulins were slightly reduced in patients with cerebral spinal sclerosis and PD, but significantly increased in Alzheimer's disease (Pankonin et al. 2009). In our study transcripts of Nrg1 were increased by 40–50% on the left side and 30–100% on the right side under both treatments, respectively. Nrg2 was slightly decreased by MPP+/saline on both sides. After MPP+/9-methyl-βC treatment Nrg2 was normalized on the left, but unchanged on the right side.

Neuropeptide Y (Npy) was slightly increased after MPP+/saline treatment on both sides and upregulated by about 50% after MPP+/9-methyl-βC treatment. Npy1r was increased by 20 and 35% on the left side by both treatments and by 50 and 130% on the right side. Npy2r was decreased by 30% by MPP+/saline treatment on the left side. MPP+/9-methyl-βC treatment revealed 35% upregulation compared to controls on the left side. The treatments increased Npy2r expression on the right side by 25 and 80%, respectively. The recovery of Npy2r is important as it has been demonstrated that Npy exerts its neuroprotective action against excitotoxicity via the Npy2r, whereas an Npy1r agonist was not protective in this model (Smialowska et al. 2009). The differing role of Npyr2 and Npyr1 in neuroprotection was also demonstrated by Xapelli et al. (2008).

Maged1, also known as Dixin-1, facilitates cell death by interaction with p75NTR. However, in the presence of Ngf it increased cell survival and neuronal differentiation via an interaction with the neurotrophin receptor TrkA, by activating the MEK (neuritogenesis) and Akt (survival) pathway (Reddy et al. 2010). Maged1 was slightly upregulated on the left side after the combined treatment. Both treatments induced an upregulation on the right side of 40 and 90%, respectively. As reported previously, Ngf was increased after MPP^+/saline and further by MPP^+/9-methyl-βC treatment in our experiments (Wernicke et al. 2010).

Fibroblast growth factor (Fgf) family members are involved in mitogenic and angiogenic activities. Winner et al. (2008) demonstrated that Fgf2 together with the epidermal growth factor enhanced cell proliferation and numbers of doublecortin-expressing neuroblasts in the subventricular zone and its migration into the striatum in a 6-OHDA animal model of PD. Jensen et al. (2008) demonstrated an improvement of survival and grafting of ventral mesencephalic precursor cells after Fgf2 treatment. Huang et al. (2009) reported on Fgf9 downregulation by MPP^+ treatment and the ability to prevent MPP^+-induced cell death of DA neurons by adding Fgf9. In our experiments the expression of Fgf2 (and to a minor extent Fgf1) was reduced on the left side of sham-operated animals. This could be an explanation for the different findings where Fgf2 was enhanced by nearly 50% by MPP^+/saline on the left side but not on the right one. MPP^+/9-methyl-βC treatment enhanced the expression only slightly on the left side, but by about 100% on the right side compared to MPP^+/saline-treated rats. The change in expression of Fgf1 was nearly identical to that of Fgf2. Fgf9 was enhanced on both sides by 40 and 30% after MPP^+/saline treatment and by 60 and 140% after MPP^+/9-methyl-βC treatment. The enhanced expression after MPP^+/saline treatment is in contrast to the findings by Huang et al. (2009). One explanation could be that in our experiment the animals had a 14-day period of recovery, in which some self-healing processes may occur, which are with respect to Fgf further supported by the findings after MPP^+/9-methyl-βC treatment.

Transforming growth factor β (Tgfb1) is a key mediator of cell proliferation, differentiation and apoptosis. It was reported to enhance survival of DA neurons and to protect against MPP^+ toxicity (Kriegelstein and Unsicker 1994; Poulsen et al. 1994). However, the inhibition of survival was also reported (Tgfb1 was nearly unchanged after both treatments on both sides). The Tgfb-induced transcript (Commissiong et al. 1997) Tgfb1i1, also known as Hic-5, seemed to mediate survival as knock-out mice displayed enhanced vascular apoptosis. A function for Tgfb1i1 in the CNS has not yet been reported. Tgfb1i1 was upregulated on both sides by MPP^+/saline by 30% and further increased by 40 and 60% after MPP^+/9-methyl-βC treatment.

The corticotropin-releasing hormone (Crh) is secreted by the hypothalamic paraventricular nucleus in response to stress. A reduction in this protein was observed in patients with Alzheimer's disease. Neuroprotective effects were reported for low concentrations of Crh in cerebellar and hippocampal cell cultures and higher concentration in cortical neurons (Bayatti et al. 2003). These effects were PKA-dependent and resulted in CREB activation and inactivation of GSK3beta. In vivo administration of Crh-enhanced neuronal survival of CA1 neurons in an ischemia

model of rats (Charron et al. 2008). In our study, Crh was upregulated by 30 and 100%, respectively, after MPP^+/9-methyl-βC treatment. Crhbp and Crhr1 were upregulated by MPP^+/saline treatment, which was further enhanced by MPP^+/9-methyl-βC treatment. Crhr2 was downregulated after MPP^+/saline by 30% and upregulated by 50% after MPP^+/9-methyl-βC treatment on the left side. The right side was unaffected.

Galanin has been demonstrated to modulate hippocampal neuronal survival and several studies point to the type 2 receptor mediating antiapoptotic effects (Elliott-Hunt et al. 2007; Hulse et al. 2011). Schauwecker (2010) reported a higher susceptibility to excitotoxic injuries and following kainate administration in Galr1 knock-out mice. In our experiment Galr1 was reduced by 30% after MPP^+/saline treatment on the left side which was normalized after MPP^+/9-methyl-βC treatment. On the right side the expression was not affected by MPP^+/saline and was enhanced by 130% after MPP^+/9-methyl-βC treatment. Galr2 was not affected on either side by MPP^+/saline and upregulated by MPP^+/9-methyl-βC treatment by 70 and 50%, respectively.

Hypocretin1 (Hcrt), also known as orexin A is a neuropeptide involved in feeding behavior, hormone secretion, sleeping behavior and arousal. Low levels of Hcrt in the CSF were associated with sleep symptoms in PD (Hag et al. 2010). Hcrt was upregulated on the left side after both treatments by 20 and 30%, respectively. On the right side it was upregulated by 90% after MPP^+/9-methyl-βC treatment. Their respective receptors, Hcrtr1 and Hcrtr2, were upregulated on the left side after combined treatment by 60 and 65%. On the right side the expression was enhanced by 60 and 30% after MPP^+/saline treatment and Hcrtr1 was further enhanced by 240% after the 9-methyl-βC treatment, whereas Hcrtr2 was only enhanced by 30%.

10.5 Transcription Changes of Chemokines, Signal Transducers, Interleukins, and Other Factors Involved in Apoptosis and Inflammation by 9-Methyl-βC

Figure 10.5 presents the transcript changes of genes involved in apoptosis, inflammation and further signal cascades. The chemokine receptor1 (Cx3cr1) protects DA neurons from toxin effects. Knock-out mice lacking this receptor developed a more extensive neuronal cell loss in a toxic model of PD and a transgenic ALS-model than their Cx3cr1-expressing littermates (Cardona et al. 2006). In our study, the receptor was marginally enhanced on the left side by both treatments and was up 60% on the right side after MPP^+/9-methyl-βC treatment. Cxcr4 is upregulated in PD and the upregulation precedes the loss of DA neurons in MPTP-treated mice (Shimoji et al. 2009). The Cxcr4 receptor, which together with its ligand Cxl12 promotes neuronal apoptosis, was enhanced 1.5- to 1.3-fold on both sides after MPP^+/saline treatment and normalized on both sides after MPP^+/9-methyl-βC treatment.

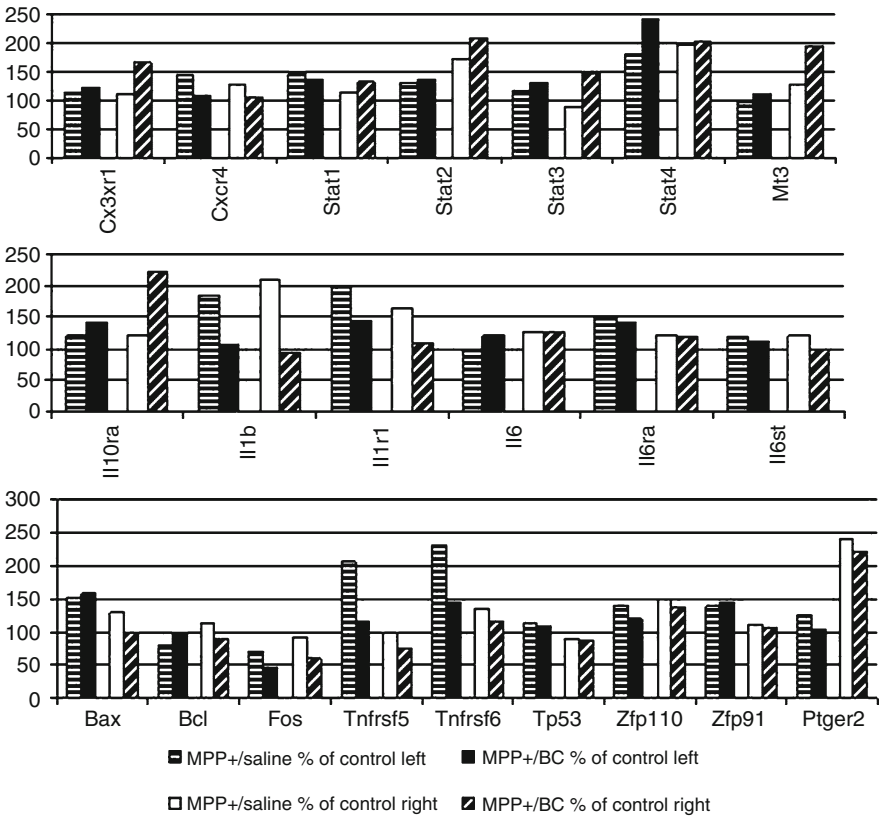

Fig. 10.5 Depicted are transcription changes of chemokines, signal transducers, interleukins, and other factors involved in apoptosis/inflammation in the left and right striatum after MPP$^+$/saline and MPP$^+$/9-methyl-βC treatment compared to sham-operated animals

Members of the signal transducer and activator of transcription (Stat) family are activated by phosphorylation in response to cytokines and growth factors. They mediate the expression of a variety of genes. IL6 is an activator of Stat1. Stat1 was upregulated by up to 50% on the left side after both treatments and on the right side after MPP$^+$/9-methyl-βC treatment. Stat2 was upregulated by up to 40% on the left side after both treatments and to 70–100% on the right side. This protein can not bind directly to DNA. It has been demonstrated that the transcription adaptor P300/CBP (EP300/Crebbp) specifically interacts with Stat2, blocking IFNα-response by adenoviruses. In null or haploinsufficient transgenic mice for Stat1 and Stat2, respectively, IFNα produced a more severe and accelerated neurodegeneration or severe inflammation (Campbell 2005). Stat3 transcription was marginally increased on the left side after both treatments and twofold on the right side after MPP$^+$/9-methyl-βC treatment. Wang et al. (2009) reported the disruption of Stat3 signaling by 6-hydroxydopamine (6-OHDA) in SH-SY5Y cells and its restoration by (−)-epigallocatechin-3-gallate which protects these cells against 6-OHDA-induced cell death.

Stat 4 transcription was increased 2- to 2.5-fold after MPP+/saline and 2.5- to 3-fold after MPP+/9-methyl-βC treatment. Stat4 is activated by IL12 and is critical in the regulation of inflammation and T-helper type 1 lineage development in the murine system. Several studies showed an association of this gene with autoimmune diseases like Sörensen syndrome, multiple sclerosis, and rheumatoid arthritis. Mt3 is a metal-binding protein with antioxidative properties. Neuronal damage was dramatically increased in Mt3-KO mice after transient cerebral ischemia (Koumura et al. 2009). The protein was unchanged on either side by MPP+/saline treatment and upregulated by nearly 30 and 50% after MPP+/9-methyl-βC treatment.

The main criterion for a cell to enter into the apoptosis pathway is the ratio between Bax and Bcl. In the left striatum the ratio was higher after MPP+/saline treatment (1.9) compared to MPP+/9-methyl-βC treatment (1.6). These findings are consistent with results reported by Zhao et al. (2010), who found a ratio of 2 in MPTP-treated mice (i.p. daily for 5 days) and a ratio of 1.6 in mice treated with MPTP, and subsequently 10 mg/kg echinacoside (i.p. daily for 14 days) 72 h after the last MPTP administration. In the right striatum the ratio was equal after both treatments (1.1). Bcl is a downstream product of CNTF signaling. As mentioned above, CNTF was enhanced after MPP+/9-methyl-βC treatment, whereas MPP+/saline resulted in CNTF downregulation. Fos, in combination with Jun is denoted transcription factor AP1, which regulates proliferation, differentiation, and transformation. Under certain circumstances AP1 induces apoptotic cell death. Fos was slightly downregulated by MPP+/saline treatment and further downregulated by MPP+/9-methyl-βC treatment. The downregulation in the left striatum with MPP+/saline treatment could be the result of a compensatory mechanism after withdrawal of the toxin. The tumor necrosis factor receptor super family members (Tnfrsf) 5 and 6 were both twofold enhanced in the left striatum by MPP+/saline and 1.5-fold by MPP+/9-methyl-βC. A reduction by MPP+/9-methyl-βC was also produced in the right striatum. Tnfrsf5 is also known as CD40 and mediates immune and inflammatory responses by increasing COX-2 and iNOS (Okuno et al. 2005). Tnfrsf6 is also known as Fas and this isoform, containing the death domain, induces apoptosis. The expression of Fas was increased in the 6-OHDA rat model of PD (Pan et al. 2007). TP 53 was nearly unchanged under all treatments. The JNK-p53-GADD45alpha apoptotic cascade mediates oxidative cytotoxicity (Choi et al. 2011). The nearly unchanged expression in the left striatum after MPP+/saline application is surprising and may be due to a mild oxidative damage, which probably had been overcome over after 14-day vehicle infusion, the time-point of tissue preparation. Zfp110, also known as neurotrophin receptor interacting factor (NRIF), is an essential mediator of apoptotic signaling in the nervous system by the p75 neurotrophin receptor. Linggi et al. (2005) demonstrated that NRIF expression is sufficient to induce cell death through a mechanism that requires p53. MPP+/saline enhanced expression by 40 and 50% on both sides which was diminished by 20% after MPP+/9-methyl-βC. Zfp91 is localized in nuclei and shows several characteristics of other transcription factors. It may play a role in cell proliferation and/or antiapoptosis. This transcript was upregulated in the left striatum by both treatments, whereas the right striatum was nearly unaffected. Ptger2 encodes the prostaglandin E2 receptor subtype 2. It is expressed by

microglia and neurons. It was demonstrated that this receptor is significantly induced in astrocytes and microglia of the SOD mouse model of ALS. After deletion of this receptor in the mouse model proinflammatory effectors were reduced (Liang et al. 2008). Furthermore, Jin et al. (2007) demonstrated a regulative role of the receptor in the clearance of aggregated α-synuclein by microglial cells. This property was upregulated in Ptger2-ablated cells and mice lacking this receptor demonstrated attenuated neurotoxicity and α-synuclein aggregation after MPTP treatment. Conversely, Carrasco et al. (2008) demonstrated a protective effect of this receptor to 6-OHDA-mediated low oxidative stress in DA neurons. This action was PKA-dependent. Therefore, it is important to determine whether this receptor is expressed by microglia or neurons. In our setting the interpretation of our data was difficult since we could not distinguish between neuron and glial cell expression. Since glia normally outnumber neurons, it is more likely that the transcript was primarily derived from glial cells. This assumption was also supported by the fact that surgery itself, which was performed on the left side, induced an increased expression in the left striatum of the sham-operated controls compared to the right side. By taking into account the left to right differences, Ptger2 was by 25% higher after MPP^+/saline treatment than after MPP^+/9-methyl-βC treatment on both sides.

IL10 has pleiotropic effects in immunregulation and inflammation. It downregulates the expression of Th1 cytokines and mediates immunosuppressive and neuroprotective effects. It is significantly increased in PD but so far not affected by treatment (Rentzos et al. 2009). IL10 gene transfer has been demonstrated to be protective in a rat model of PD (Johnston et al. 2008). In our study, the transcripts of IL10 were near the limits of detection in the striatum. However the IL10 receptor alpha was upregulated on both sides by up to 20% after MPP^+/saline treatment and up to 40 and 120% on the left and right side, respectively, after MPP^+/9-methyl-βC treatment. The proinflammatory interleukin IL1b was enhanced by 80–100% after MPP^+/saline treatment on both sides and normalized after MPP^+/9-methyl-βC treatment. This is consistent with the expression pattern of its receptor IL1r1 with an upregulation of 100 and 60% by MPP^+/saline and a reduced upregulation on the left side after MPP^+/9-methyl-βC treatment and normalization on the right side. IL1b has been demonstrated to be elevated in the MPTP rat model of PD (Bian et al. 2009). It was shown to be elevated in the striatum and the SN of PD patients. IL6 and IL6 signal transducers were only marginally changed. IL6 induces transcriptional inflammatory response by the IL6 receptor alpha, which was upregulated by nearly 50% on the left side and by nearly 20% on the right side after both treatments.

Nurr1, Pitx3, Bmp2, and BMP4 were not changed. This is consistent with the fact that these factors are important in the maturation of DA neurons. In our setting it is more likely that the rescue depends on the protection of mature neurons which had been impaired by MPP^+. In the human neuroblastoma cell line SH-SY5Y 9-methyl-βC induces the expression of Pitx 3 and BMP 2, which is in line with the differentiation of these cells from an immature to a mature neuronal cell (Wernicke et al., unpublished results).

Taken together, the treatment with 9-methyl-βC resulted in an upregulation of several transcripts that are known to play an important role in cell survival and neu-

ronal differentiation. Although, the individual differences in expression of numerous transcripts were not significant, the consistent upregulation of protective factors and downregulation of inflammatory and apoptosis-inducing factors reveal a pattern that is unlikely to be the result of chance. Nevertheless, further investigations are necessary to confirm these findings. Notably, our investigation represents the results of a single time-point of an in vivo model of PD. The unpublished in vitro studies demonstrate that the extent of the expression of individual genes change over time and the composition of a group of genes with similar functions changes over time as well. The expression changes in such a huge number of genes points to the involvement of an upstream pathway.

This pathway may be triggered by one of the high-affinity binding sites described by Lichtenberg-Kraag et al. (1997). [^{3}H]Norharman binds to SH-SY5Y membranes with a K_D of ~500 pM and of ~ 6 nM. The simple βCs norharman and harman activate neurons by low concentrations/ doses and inhibit them by somewhat higher ones which is consistent with the findings of the binding experiments (in vitro neurophysiology, Ergene and Schoener, 1993, in vivo microdialysis, Sällström-Baum et al. 1996). Pharmacological characterization of the [^{3}H]norharman binding sites revealed displacement of the ligand by βCs, to a weaker extend by indoleamines, but not by opioids, muscarinic receptor agonists, metabotropic glutamate receptor agonists or several peptide neurotransmitters. Inositol phosphate accumulation was only slightly affected by the βCs. However, the action of carbachol was clearly facilitated in a dose-dependent and pertussis toxin-insensitive manner. Pretreatment of the cells with *Clostridium difficile* toxin B blocked the facilitating effect of the ßCs by concentrations which did not affect the action of carbachol alone. This suggests that low molecular weight GTPase proteins, specifically RhoA and/ or Cdc-42, are involved in the facilitating action of the βCs. Further experiments demonstrated βC-induced generation of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2), the key component in the activation of phosphoinositide-phospholipase C. These interactions and the subsequent cascade which finally activates phosphorylation of CREB are depicted in (Fig 10.6).

The ßCs may activate CREB by a second cascade. Experiments aimed at identifying binding proteins of βCs revealed Raf kinase inhibitor protein (RKIP) as a target protein (Bonnet et al. 2004). RKIP is phosphorylated by PKC and dissociates from the Raf/ MEK complex to the G-protein kinase 2 which is inhibited by phosphorylated RKIP. This prevents reduction of GPCR activity and prolongation of cAMP formation. cAMP activates PKA from which the catalytic unit diffuses into the nucleus and phosphorylates CREB (Hellmann et al. 2010). Finally, neurotrophins activate the Ras/Raf/MEK/ERK cascade leading to the phosphorylation of CREB. The activation of these cascades may explain the remarkable stimulation of CREB phosphorylation observed after exposition of SH-SY5Y human neuroblastoma cells for 48 h applying the dual luciferase method (4fold by 30μM and 8-fold by 70 μM 9-methyl-βC, unpublished results). The activation of these cascades may occur partly consecutively and may be compensated by other intracellular mechanisms. Many of the neuroprotective and neurorestorative actions of the βCs may be based on these processes.

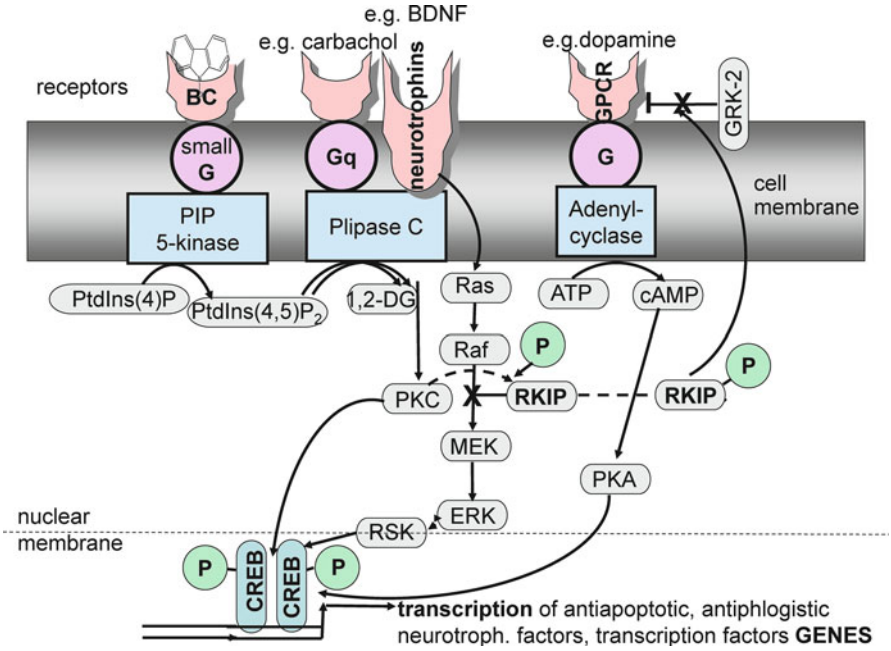

Fig. 10.6 Putative ßC receptor activating several intracellular cascades. Abbreviations: PIP 5-kinase, phosphatidylinositol 5-kinase; PtdIns(4)P, phosphatidylinositol(4)phosphate; 1,2-DG, 1,2 diacylglycerol; Gq, heterotrimeric GTP-binding protein of the GQ family

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Chapter 11
Prospects for New Treatment Options in Neurodegenerative Diseases

Hans Rommelspacher

Contents

Abstract Animal studies clearly demonstrate that either dying neurons are revived or remaining healthy neurons sprout new processes in response to neurotrophic factors. Thus, in view of their restorative potential, specific alignments of neurotrophic factors may be called the "natural repair system" for damaged neurons. However, several serious hurdles remain to be overcome before the neurotrophins themselves are applicable for the treatment of patients with Parkinson's disease (PD) and other neurodegenerative disorders. Alternatively, the antioxidant, the efficiency of the respiratory chain improving, antiphlogistic, anti-apoptotic, prodifferentiating, and neurotrophin-activating β-carbolines are promising small molecules with neuroprotective and even restorative potential. They may become alternative compounds for the therapy of PD and other neurodegenerative diseases in the foreseeable future.

Keywords Neurotrophins • β-Carbolines • Restoration • Parkinson's disease • Antioxidant • Respiratory chain • Antiphlogistic • Apoptosis • Differentiation

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11.1 Pathogenesis of Neurodegenerative Disorders

The pathogenesis of neurodegenerative disorders appears to be multifactorial with genetic predisposition, exogenous factors including environmental toxins, heavy metals, and viruses as well as aging being important factors in disorder initiation and progression. Several factors have been elaborated underlying Parkinson's disease (PD), the focus of our studies. Cumulative evidence supports an "oxidative stress hypothesis" for nigral dopamine (DA) neuron loss in PD. Essentially, the substantia nigra (SN) has a high metabolic rate combined with both a high content of oxidative species including DA and DA-derived reactive oxygen species (ROS), neuromelanin, polyunsaturated fatty acids and iron, and a low content of antioxidants especially glutathione, the major cellular redox buffer used to counteract oxidative stress. Thus, oxidative stress can easily overwhelm the natural defenses and initiate apoptosis of DA neurons. One important feature of this hypothesis is the active, self-perpetuating cycle of chronic inflammation that includes microglia activation, increased chemokine and cytokine production, and nitric oxide (NO) and hyperoxide (syn. superoxide) production leading to neurodegeneration (Hald and Lotharius 2005). Therefore, trapping free radical oxidants and inhibition of the glial reaction and inflammatory processes represent attractive therapeutic targets to combat the disease.

Neurotoxins, such as 1-methyl-4-phenyl-pyridinium (MPP^+) and rotenone inhibit the respiratory chain in mitochondria. This results in the excess production of ROS, ATP depletion, and initiation of cascades of cellular disturbances that include DNA damage and activation of cell-death-related pathways including apoptosis. NO contributes to DA neuron death through mechanisms that are not completely understood, but are likely to involve nitration of α-synuclein and nitrosylation of parkin. Evidence continues to accumulate that the cycle of neuroinflammation triggered by these neurotoxins may continue to progress long after the initial insult abates.

11.2 Existing Therapies for Parkinson's Disease

Moderate disease management can be achieved through dopamine (DA) replacement therapy. Such drugs include the DA precursor levodopa, DA receptor agonists, and inhibitors of the DA degrading enzymes catechol-*O*-methyltransferase and monoamineoxidase, specifically subtype B inhibitors. However, all these pharmacological interventions have side effects and limited efficacy. A number of studies have indicated that levodopa itself may be neurotoxic and contribute to the progression of PD.

Since PD results primarily from loss of a very specific population of DA neurons in the SN pars compacta, another potential therapeutic strategy involves cell replacement therapy with fetal ventral mesencephalon grafts or adult and embryonic stem cells. Conceptual and clinical problems have been encountered in the development

of cell-based treatments including low graft survival, poor engraftment, and limited reestablishment of neural circuits leading to functional restoration.

Postmortem examination of SN revealed massive astrogliosis, the presence of activated microglia, and elevated levels of inflammatory cytokines (Mogi et al. 1994). Therefore, an emerging area of interest involves strategies to inhibit the glial reaction and act to sustain the cycle of microglia-derived oxidative stress (Barcia et al. 2003; Hirsch et al. 2005; Imamura et al. 2003). A recent prospective study found that the incidence of sporadic PD among chronic users of over-the-counter nonsteroidal anti-inflammatory drugs was 45% lower compared to that of age-matched nonusers (Chen et al. 2003).

One important apoptosis effector molecule is caspase-3 which is overexpressed in PD (Hartmann et al. 2000). Pramipexole increased the production of Bcl2 which blocks apoptosis (Kitamura et al. 1998). Furthermore, that targeting the c-jun kinase signaling pathway, which is often activated by cellular stress and correlated with neuronal death, has shown inhibitors to be neuroprotective and being investigated for use in stroke and PD (Kuan and Burke 2005; Silva et al. 2005).

One other area of interest concerns the neurotransmitter glutamate whose neocortical input to the SN is hyperfunctional in PD. Certain glutamate receptor antagonists have anti-parkinsonian activity (Chase et al. 2000). A further area concerns the possible role of neurotrophic factor depletion in the etiology or progression of PD. Conditional ablation of glial cell line-derived neurotrophic factor (GDNF) in mice resulted in a progressive hypokinesia and pronounced death of catecholaminergic neurons (Pascual et al. 2008). It has been proposed that age-related loss of GDNF in the nigrostriatal system may be associated with increased vulnerability of DA neurons to neurotoxic and/or oxidative insults. Early promising findings with GDNF were followed by disappointing efficacy results perhaps due to differences in dosage, delivery mode, and catheter size (Kotzbauer 2006). Neurturin, a member of the GDNF family, which is expressed endogenously in the nigrostriatal system, was found to promote the survival of DA neurons both in vitro and in vivo with similar efficacy as GDNF. Phase I clinical trials to test the efficacy of neurturin in PD patients demonstrated the safety, tolerability, and potential efficacy of AAV-neurturin (Marks et al. 2008). The Phase II trial failed to demonstrate improvement of primary end point (Ceregene 2011).

Several other neurotrophic factors are involved in survival of DA neurons. BDNF is synthesized by DA neurons in the SN of rats and the expression level is decreased in the SN of PD patients (Howells et al. 2000). Animal studies demonstrated the restorative potential of BDNF. However, clinical trials of intrathecal infusion of recombinant BDNF have been negative and caused severe side effects (Ochs et al. 2000). A neurotrophic factor with selective effect on the survival of rat primary DA neurons is mesencephalic astrocyte-derived neurotrophic factor (MANF; Petrova et al. 2003). MANF provided DA neuroprotection when administered 6 h before and 4 weeks after administration of the neurotoxin 6-hydroxydopamine (Voutilainen et al. 2009). Conserved dopamine neurotrophic factor (CDNF) is a vertebrate-specific paralogue of human MANF. CDNF is highly produced in various brain regions, including cortex, hippocampus, SN, cerebellum, and locus coeruleus in adult mice.

When administered into the striatum from rats 4 weeks after 6-OHDA, CDNF was able to restore DA function and prevent degeneration of DA neurons in the SN (Lindholm et al. 2007). Thus, data are available in animals that either dying neurons are revived or the remaining healthy neurons sprout new processes in response to neurotrophic factors. These findings strongly suggest that the most promising approach for the treatment of patients with Parkinson's disease and possibly other neurodegenerative disorders involves the neurotrophins.

11.3 The Neuroprotective and Neurorestorative Potential of β-Carbolines

On the background of these findings we explored the prospects of our drug development studies for the treatment of PD. Several promising findings indicate the therapeutic potential of some of the β-carbolines (βCs).

Concerning the "oxidative stress hypothesis" for nigral DA neuron loss, the βCs target several factors. All βCs can be considered as molecules with significant antioxidant properties. First, this is for purely chemical reasons because of the reactivity of the indole part toward oxygen-derived radicals and of the pyridine part of tetrahydro- and dihydro-βCs, as well as hydroxylation to phenolic derivatives. It is notable and not generally recognized that βCs are present in many foodstuffs, for example, in fruit juices that contain two different antioxidative ingredients: ascorbic acid and the βCs. Based on the in vitro results of Herraiz and Galisteo (2003) and assuming that absorption and bioavailability of the βCs are much better than those of the highly hydrophilic ascorbic acid, it can be concluded that the βCs represent the more relevant class of antioxidants in fruit juices and possibly other foodstuffs (see Chapter 9). A second action with antioxidant consequences is the potent inhibition of monoamine oxidase (MAO) A and B by some βCs. Total MAO activity is present in the brain in large amounts and it is only apparent when both isoenzymes are inhibited that the DA level increases. In addition, the amount of MAO B is increased in PD due to gliosis and in smokers due to a compensatory mechanism for the inhibition of the enzyme caused by smoke constituents (Launay et al. 2009). Both isoenzymes should be inhibited to a certain extent to achieve neuroprotection by decreasing, e.g., the detrimental ROS production by the enzyme activity. The remarkable correlation between the levels of the βCs, norharman (inhibitor of MAO B) and harman (inhibitor of MAO A) in tobacco smoke and the proportion of the inhibited isoenzymes in the human brain found in PET studies, strongly support the view that these two βCs are reasonable candidates responsible for the inhibition of MAO A and B in human brain and other organs from active smokers. The large field study showing smoking subjects to have a reduced risk for developing PD support the view of preferential neuroprotection of DA neurons by these βCs, a preference which has also been found previously in electrophysiological and microdialysis experiments in rats. A third action concerns the reduced formation of ROS by 9-methyl-βC (other βCs have not yet been investigated). This action is due to

improvement in the efficiency of the respiratory chain in mitochondria, the main source of ROS (see chapter 8). ROS induces a glia cell reaction and inflammatory processes in the tissue. In vitro and in vivo studies reported anti-inflammatory effects of 9-methyl-βC not only by reducing the amount of cytokines but also by reducing the microglial reaction (see chapter 10).

The most promising action of βCs with respect to neuroprotective and even restorative effects in PD and other neurodegenerative disorders is the induction of the transcription of neurotrophic factors. Unpublished data strongly suggest that the activation primarily involves stimulation of the protein kinase A-CREB (cAMP-response-element-binding-protein) signaling pathway (eightfold stimulation in human neuroblastoma cells after 7 days of exposition). CREB is the final transcription factor of several intracellular cascades including those stimulated by AMPA and NMDA glutamate receptors. In addition, CREB and its cofactor CREB binding protein are regarded as canonical TH gene regulating transcription factors (Kim et al. 1993; Ghee et al. 1998; Lewis-Tuffin et al. 2004).

Several neurotrophins are produced by DA neurons and others support survival specifically of DA neurons. Most of the attention within the last 10 years or so has focused on two factors: GDNF and neurturin. These factors seem to be the ones that work the best on dopaminergic neurons. Other trophic factors extensively explored for their therapeutic potential in PD include other neurotrophins, such as CDNF and MANF. One of the biggest challenges with developing neurotrophic factors as a treatment is getting them into the brain. Trophic factors are proteins, and proteins are excluded from the blood–brain barrier (BBB). So, administering the factors orally or by intravenous injection is not an option at this point. Studies are underway to explore intranasal therapy to deliver GDNF directly to the brain, bypassing the BBB.

Surgery is the most advanced method for delivering neurotrophins. In one type of surgery, the protein is infused into the brain. A concern is that a protein could trigger an immune reaction. In the other surgical method, a gene that encodes for the protein is delivered within a viral vector to the brain (gene therapy).

With viral delivery, the gene for the neurotrotrophic factor is taken up by the patient's cells and the protein is, in theory, produced indefinitely. Currently, there is no way to regulate the gene after it is delivered if an adverse event occurs. Protein infusion and gene therapy approaches work very well in animal models of PD, but clinical trials produced disappointing results. Therefore, the strategy of applying a small molecule which passes the BBB easily and activates the transcription of neurotrophic factors seems to be promising. By activating the natural mechanisms involved in gene regulation there is no concern with respect to immune reactions. A further advantage of βCs is the preference for DA neurons which has been demonstrated in electrophysiological and in vivo microdialysis experiments. Our lead compound 9-methyl-βC activated a number of neurotrophins in the striatum in vivo (Wernicke et al. 2010) and in primary mesencephalic culture from embryonic mice (Hamann et al. 2008; Polanski et al. 2011). Unpublished studies with human neuroblastoma cells demonstrate that the stimulation of the transcription of neurotrophic factors changes over time. This concerns not only the amount of single factors but

also their absolute presence. Thus, regulation seems to depend on certain, yet unknown conditions.

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