

G. Di Giovanni · V. Di Matteo
E. Esposito *Editors*

Birth, Life and Death of Dopaminergic Neurons in the Substantia Nigra

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in the Substantia Nigra

Edited by
G. Di Giovanni, V. Di Matteo, E. Esposito

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To Giulio Di Giovanni, who suffered from Parkinson's disease

Preface

The aim of this supplement of *The Journal of Neural Transmission* is to provide the reader with a unique and timely multidisciplinary synthesis of our current knowledge of the anatomy, pharmacology, physiology, and pathology of the substantia nigra pars compacta dopaminergic neurons. This is most probably one of the most investigated groups of neurons in scientific literature since the discovery of dopamine deficiency in Parkinson's disease by Hornykiewicz and his collaborator Ehringer at the end of the 1950s. With this in mind, we offer as varied a picture as possible, by including exhaustive reviews as well as original research papers covering different points of view, together with different aspects of the life cycle of dopamine neurons from their birth to death. New evidence has recently emerged thanks to the development of new techniques in molecular biology, genetics, single-cell and membrane physiology, clinical neurology, and *in vivo* brain imaging. The selection starts by reviewing the ontogeny of nigrostriatal dopamine neurons. Thereafter, single chapters explore dopaminergic neuron functions from the typical motor to the other variegated cognitive ones examined from different perspectives. In the section dedicated to death of dopaminergic neurons, the ways in which the neurodegenerative process begins and progresses are singled out in different chapters. Finally, new therapeutic approaches such as immunization, gene therapy, and stem cells and cell replacement therapy and the latest evidence of a possible *de novo* neurogenesis in the SNc are reviewed. The paramount role of dietary factors in counteracting DA degeneration is also examined. Indeed, promoting healthy lifestyle choices such as a Mediterranean diet might be the key to reducing the risk of Parkinson's disease.

It is reasonable to hope that new research findings will disclose to us both the secrets of the organization of the substantia nigra pars compacta and clues to its vulnerability in Parkinson's disease.

With this supplement of *The Journal of Neural Transmission*, we have tried to bridge basic science and clinical practice and help to prepare the reader for the next few years, which will surely be eventful in terms of the progress of dopamine research. Therefore, pharmacologists, neuroscientists, and students will find this important work useful.

While covering the latest research, for obvious reasons, this volume cannot be exhaustive and we are sorry indeed that it has been impossible to include a number of authors of obvious merit. The selection is intended, indeed, to be merely a very varied foretaste of contemporary research on the subject.

The editors thank all the authors who have responded very willingly and contributed their time and expertise in preparing their individual contribution to a consistently high standard. Our warmest thanks go to Silvia Schilgerius, Springer publishing editor, who believed in the potential of this book and the importance of the messages it conveys, and Katrin Stakemeier (Springer-Verlag), who has helped to drive the book's development and eventual publication. Our thanks go to everyone who has, in same way, contributed to the realization of this book, notably Samantha, Barbara, and Christopher. And finally, we are grateful to Dr Clare Austen for a very insightful and helpful reading and for reviewing the English style of these manuscripts.

G. Di Giovanni, V. Di Matteo, E. Esposito

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Chapter 1

Ontogeny of Substantia Nigra Dopamine Neurons

Orme R, Fricker-Gates RA, and Gates MA

Abstract Understanding the ontogeny of A9 dopamine (DA) neurons is critical not only to determining basic developmental events that facilitate the emergence of the substantia nigra pars compacta (SNc) but also to the extraction and de novo generation of DA neurons as a potential cell therapy for Parkinson's disease. Recent research has identified a precise window for DA cell birth (differentiation) in the ventral mesencephalon (VM) as well as a number of factors that may facilitate this process. However, application of these factors in vitro has had limited success in specifying a dopaminergic cell fate from undifferentiated cells, suggesting that other cell/molecular signals may as yet remain undiscovered. To resolve this, current work seeks to identify particularly potent and novel DA neuron differentiation factors within the developing VM specifically at the moment of ontogeny. Through such (past and present) studies, a catalog of proteins that play a pivotal role in the generation of nigral DA neurons during normal CNS development has begun to emerge. In the future, it will be crucial to continue to evaluate the critical developmental window where DA neuron ontogeny occurs, not only to facilitate our potential to protect these cells from degeneration in the adult brain but also to mimic the developmental environment in a way that enhances our ability to generate these cells anew either in vitro or in vivo. Here we review our present understanding of factors that are thought to be involved in the emergence of the A9 dopamine neuron group from the ventral mesencephalon.

Keywords Dopamine neurons • Ontogeny • Parkinson's disease • Proteomics • Substantia nigra

Why Study the Ontogeny of Substantia Nigra Dopamine Neurons?

The ontogeny of the A9 dopamine (DA) cell group would be of little interest if it were not for the human neurodegenerative disorder Parkinson's disease (PD). Epidemiologically, idiopathic PD is a worldwide disease, affecting approximately 1–2% of the population over the age of 60 (von Campenhausen et al. 2005). Idiopathic PD was first formally described by James Parkinson (in his now famous; Essay on the Shaking Palsy) as “paralysis agitans”, a disorder of “involuntary tremulous motion, with lessened voluntary muscle power” (Parkinson 1817). Historically, there was little or no advancement on this original description, or any additional understanding of the underlying causes for the disease, for nearly 50 subsequent years (Louis 1997). It was then that a French physician, Jean-Martin Charcot, began giving much more detailed descriptions of the motor abnormalities associated with the disease (Goetz 1986). Charcot not only recognized very precise oscillation rates in the resting tremors of PD sufferers but also seemed to indicate that the disorder was distinctly of nonpyramidal origins, arguing against the use of the word “paralysis” (used by Parkinson) to describe the disease (Goetz 1986).

It was nearly a century before the work of Arvid Carlsson began the process of identifying the underlying physiological and anatomical regions of the brain affected by PD. Carlsson's work in the late 1950s showed the effects that monoamine depletion had on the movement of laboratory animals, indicating not only that dopamine itself may be a neurotransmitter in its own right but also that it may play a crucial role in the neurological process that enabled movement in mammals (Carlsson et al. 1957). In his seminal studies, Carlsson showed that rabbits rendered motionless by monoamine depletion were “revived” by injections of the DA precursor L-dihydroxyphenylalanine (l-dopa: for review see (Abbott 2007). L-dopa, itself, was not a wholly new substance or without any use in neurological disorders. As early as 1913, l-dopa was being synthesized from plants,

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although there was, at that time, no known application for the DA precursor. And, even before this, plants (such as *Mucuna pruriens* or “velvet bean”) that (unknown at the time) contain naturally high amount of l-dopa were being used to treat a variety of disorders (for review see (LEES 1986). By the early 1970s, l-dopa was being marketed by the pharmaceutical industry for the treatment of PD, with clinical trials reporting relatively effective treatment of the main manifestations caused by disruption of the nigro-striatal circuit; namely bradykinesia/akinesia and resting tremors. Today, DA supplementation is the gold standard for treating the disorder in its early stages; however, it has a limited period of efficacy (~10 years).

It took the seminal work of Hornykiewicz in the 1960s to identify specific areas of the brain affected by PD and how it is the degeneration of the circuit that emerges from the A9 DA cell group that is the principal cause of many of the movement abnormalities seen in PD (Hornykiewicz 2008). Hornykiewicz achieved this by first noting that there was an unusually high DA content in the striatal region of the human brain: too high for the monoamine to be a mere precursor for noradrenaline. More importantly, Hornykiewicz revealed that DA levels were unusually low in post-mortem samples of the PD striatum but was relatively normal from the same region of Huntington’s disease specimens (where there is a marked decrease of neurons within the caudate and putamen). Hornykiewicz rightly deduced from this that the source of DA in the striatum must be coming from cells/neurons that were outside the striatal region. Although this was hotly debated at the time, it is now generally agreed that these studies marked the beginning for identifying the nigro-striatal system (and the A9 DA cell group) as the principal central nervous system (CNS) component involved in the movement abnormalities associated with PD (for review see Hornykiewicz 2008).

Very shortly after these historical findings, interest in the field of neural transplantation began to (re)emerge. After the experimental technique had largely been abandoned for nearly half a century, two groups in the 1970s (Bjorklund and Stenevi 1971; Das and Altman 1971) began exploring the potential of achieving surviving, functional grafts in laboratory animals. By the end of the 1970s, these groups had begun exploring how neural transplantation might be used to treat neurological disorders, PD in particular (Bjorklund and Stenevi 1979; Das et al. 1979). Because the combined findings of the effectiveness of l-dopa therapy for PD and the anatomical localization of cells that degenerate in PD preceded this revolutionary therapeutic ideal, it was possible to quickly identify the region of the embryonic brain (i.e., the ventral mesencephalon) where a source for DA neurons could be used for a cell replacement strategy in advanced stages of PD. In the past two or three decades,

efforts to establish a suitable cell replacement strategy for the disease have met with good success, although there appears to be some inconsistency in technique, and ultimately, results. Crucially, one potential factor that may lead to variable results in primary cell transplants is the great specificity of the A9 DA cell group (see the following section). Recent work (Thompson et al. 2005, 2006) has shown a striking precision of DA cell integration after transplantation, where only cells that will give rise to the A9 DA cell group, and not the DA cells from the adjacent ventral tegmental area (VTA), are effective at restoring functional connectivity in the striata of animal models of PD.

A prevailing obstacle to a more widespread use of DA cell replacement in cases of PD, though, is the acquisition of a suitable number of donor cells (Correia et al. 2005). From the beginning of the cell replacement strategy, it was noted that dissected primary neurons would have to be extremely specific (e.g., in terms of age, cell viability, etc.) to establish effective grafts in PD patients and would necessitate large amounts of fetal neural tissue (five or more embryonic donors per patient) to achieve a functional outcome in patients. While current efforts are focusing on maximizing the viability and the yield of primary neurons extracted from the SNc region of donor embryos (Brundin et al. 2000; Torres et al. 2007), other studies explore the generation of neural stem cell lines (Reynolds et al. 1992), which would (in addition to addressing the problem of the number of neurons needed for cell replacement) negate the logistical and ethical problems associated with primary cell transplantation (Paul et al. 2002). If cells of a neural origin could be induced to divide, then this would circumvent the need to use large numbers of embryos to generate appropriate neurons for transplantation. Initially it was thought that neural cells could be generated from developing ventral mesencephalon (VM), due to the fact that these cells would be phenotypically very close to developing DA neurons and would therefore require little manipulation to generate fully mature neurons. Furthermore, if neural stem cells could be generated from the adult substantia nigra, then these might be available to be modified *in situ*, in the PD patients’ own brains. In addition to this, the identification of embryonic stem cells (ES cells) (Evans and Kaufman 1981), and, more recently, their isolation from human embryos (Thomson et al. 1998), has opened up alternative possibilities for establishing cell lines that could be used to replace DA neurons lost to PD. To date, however, there has been mixed success in transforming either neural or embryonic stem cells into functional DA neurons (Bjorklund et al. 2002; Carvey et al. 2001). The major problems include either inability to efficiently differentiate neural stem cells to large numbers of DA neurons or to differentiate ES cells into the specific A9 lineage. Many of the successful studies have required the genetic manipulation of stem cells

(Kim et al. 2002), and this technique brings its own practical and ethical problems when considering transplantation of these cells into patients.

So why study the ontogeny of the A9 DA neuron group? Well, detailed ontological information of A9 DA neurons is of certain interest to both the primary and stem cell field, because: (1) the viability and numbers of primary DA neurons extracted from embryonic donors may be enhanced if the cells are removed at a period in development when their fate is committed, but the cells have few or no mature neuritic processes (which may compromise the cells' viability during dissection and preparation for transplantation); and (2) efforts to generate stem cells that can differentiate into specifically SNc-like DA neurons may be enhanced by both the potential to extract VM cells that have the capacity to divide but a certain commitment to the dopaminergic neuron fate, and the knowledge of the spatial and temporal generation of the SNc neurons in relation to other cellular and molecular constituents in the developing mesencephalon (Arenas 2005). In essence, it is felt that an understanding of the emergence of this small group of neurons would not only be valuable toward maximizing their viability and function for primary cell transplantation in PD but would also accelerate progress toward producing a suitable cell line for a more widespread use of the neural replacement strategy.

Emergence of the A9 Group

Studies focused on determining the emergence of substantia nigra DA neurons during embryonic development (sometimes called "cell birthdating") have been conducted for many years in laboratory rodents. Studies using autoradiographic (Altman and Bayer 1981), bromodeoxyuridine (BrdU), and/or nonstage specific phenotypic markers (e.g., tyrosine hydroxylase; TH) initially indicated that midbrain DA neurons arise within the ventral mesencephalic flexure of rats sometime around embryonic day (E)14 (Hanaway et al. 1971; Lauder and Bloom 1974; Sinclair et al. 1999; Marti et al. 2002). Although this stage of rat development is difficult to correlate with human development, it is thought that it corresponds to approximately 50 days post conception in humans (Clancy et al. 2007). However, the timing of A9 neurogenesis in rodents and humans is not without controversy. Although earlier studies suggested that peak neurogenesis of A9 DA neurons occurs around E14 in the rat, recent studies indicate that many VM DA neurons may be generated well before this stage of development. First, immunocytochemical staining for TH in the embryonic VM revealed that there are numerous TH-immunopositive (TH+) DA neurons in this region as early as E12 and that a substantial number display

axonal projections that extend more than 1 mm toward the ventral forebrain by E14 (Voorn et al. 1988; Gates et al. 2004). Other work, using BrdU / TH double immunohistochemical analysis, seems to indicate that peak neurogenesis may occur as early as E12 in the rat (Gates et al. 2006). If this proves true, then the equivalent stage in humans could be as early as 40 days post conception (Clancy et al. 2007); a significant shift in the timing of cell extraction for transplantation or stem cell derivation.

What is agreed is that midbrain DA neurons arise along the ventral mesencephalic flexure and consist of the A9 neurons of the substantia nigra and the A10 group of the ventral tegmental area. The two sets of neurons appear at approximately the same developmental time point in close proximity to two major signaling centers: the midbrain–hindbrain boundary (MHB) and the floor plate of the ventral midline of the mesencephalon. These two sites control development through production and secretion of many of the diffusible signals that contribute to specifying the terminal progeny of cells within the VM region. With this in mind, both the A9 and A10 DA cell group may be regulated by the same signaling cascade; therefore, establishment of these two signaling centers is critical to proper midbrain development. However, the two groups of DA neurons in the midbrain can be distinguished by molecular and physiological traits specific to each individual subgroup (Engele and Schilling 1996; Marin et al. 2005; Neuhoff et al. 2002; Puelles and Verney 1998; Puelles and Rubenstein 2003; Smidt and Burbach 2007; Smidt et al. 1997, 2006; Verney et al. 2001). These differences could indicate further specification of the individual groups of neurons and could ultimately be the basis behind specific vulnerability of the substantia nigra pars compacta (SNc) neurons in PD (Smidt and Burbach 2007). Additionally, as the two groups of neurons innervate different areas of the brain and have different functions, it is likely that different stimuli are responsible for the individual target recognition.

Terminally differentiated DA neurons can be characterized by the expression of proteins involved in neurotransmitter production and transport, such as tyrosine hydroxylase (TH), vesicular monoamine transporter 2 (VMAT2), DA transporter (DAT), and l-aromatic acid decarboxylase (AADC). Mature neurons, however, are more accurately characterized by the expression of paired-like homeodomain transcription factor 3 (Pitx3), which is present in all mature DA neurons (Smidt and Burbach 2007; Smidt et al. 1997). The specification of the DA neurons follows a complex cascade of protein signals through several key stages. First, patterning events define both midbrain territory along the anterior–posterior axis and the ventral region of the ventral–dorsal axis. Second, a pool of progenitor cells is produced. These cells must maintain a pluripotent state throughout the development of the dopaminergic neurons

and be able to respond to the signaling proteins controlling specification. DA neurons are then produced from these progenitor cells in response to signalling molecules. Finally, the newly formed neurons must mature to become fully functional and integrate into the CNS.

Defining the Ventral Midbrain Territory

Early patterning events are responsible for segregating the neural tube into distinct regions, such as forebrain, midbrain, and hindbrain. During development, these regions can be characterized by the expression of certain marker proteins that often play a role in defining the territories. Orthodentical homeobox 2 (*Otx2*) is an early marker of forebrain and midbrain tissue; while gastrulation brain homeobox 2 (*Gbx2*) is expressed at the same time in hindbrain in the developing neural tube. The expression of the two homeobox proteins is required for a proper development of both regions. At the boundary of the two expression domains, the mid–hindbrain boundary (MHB) is formed (Fig.1) (Millet et al. 1999; Broccoli et al. 1999). Important proteins in the specification of DA neurons such as Fibroblast Growth Factors (FGFs) and members of the Wnt family are expressed by cells in this region.

The ventral floor plate of the midbrain is another crucial signaling center for the specification of DA neurons. Many proteins are expressed in, and secreted from, cells in this region, including Sonic hedgehog (Shh), a ventralizing morphogen required for DA neuron specification (Blaess et al. 2006). The overlapping expression of Shh from floor plate cells and FGF8 from the MHB (starting at approximately E8 in mouse) appears to specify the precise region from which mature dopaminergic neurons of the midbrain will arise (Fig.2) (Roussa and Kriegstein 2004; Ye et al. 1998).

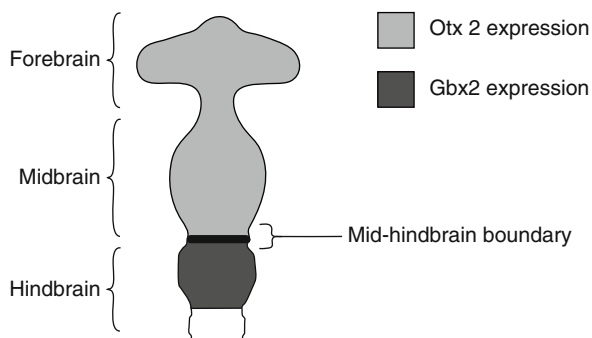


Fig. 1 Control of MHB positioning by *Otx2* and *Gbx2* expression. *Otx2* expression defines midbrain and forebrain territory, while *Gbx2* is expressed in, and defines the hindbrain. The boundary between these two expression domains defines the MHB

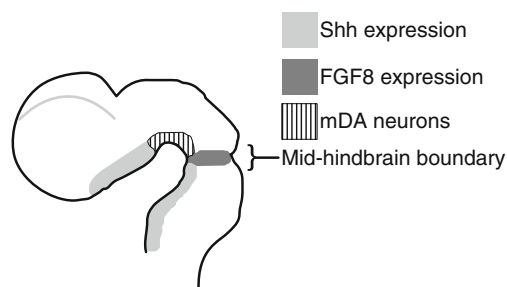


Fig. 2 The location of the DA neurons is determined by overlapping regions of FGF8 and Shh expression. FGF8 is a growth factor secreted from the anterior mid-hindbrain boundary, while the morphogen Shh is expressed in ventral locations along the neural tube

Producing and Maintaining a Progenitor Pool

Once the ventral midbrain has been defined, a pool of competent progenitor cells must be produced and maintained, from which DA neurons can be derived. This pool of cells persists throughout development and maintains a proliferative capacity, as well as the ability to differentiate into a specific neural phenotype in response to signaling mechanisms. Many signals contribute to the production of this pool of cells and also to maintaining their pluripotent nature. The earliest expression of genes associated specifically with the development of DA neuron progenitor cells is seen at approximately E8–E8.5 in mouse. These include the paired box genes *Pax2* (Puschel et al. 1992) and *Pax5* (Adams et al. 1992; Asano and Gruss 1992; Urbanek et al. 1994); the transcription factors *En1* and *En2* (Davis and Joyner 1988) and the secreted signaling molecule *Wnt1* (Rowitch and McMahon 1995). The interplay between these secreted proteins and transcription factors is crucial for the future development of DA neurons from the progenitor pool. Interestingly, the role of many of these effectors appears to continue far beyond the specification of a progenitor pool, into future maturation and maintenance of differentiated DA neurons.

Sonic Hedgehog (Shh)

Ventral patterning of the neural tube is controlled at least in part by the morphogen Sonic hedgehog (Shh). It is expressed along the entire rostro-caudal length of the ventral neural tube during development. In spinal cord, in addition to exerting a ventralizing effect, Shh regulates the expression of a set of homeodomain transcription factors in progenitor cells (Briscoe et al. 2000), which, through activation of downstream factors, results in neural subtype specification and spatial patterning (Briscoe et al. 2000; Jessell 2000; Muhr et al. 2001; Pierani et al. 1999).

This established mechanism in spinal cord development may be replicated throughout the ventral neural tube, including the midbrain.

During early development of the midbrain, Shh is necessary and sufficient to specify a ventral cell fate in progenitor cells (Hynes et al. 1995). Analysis of *Shh*^{-/-} mice has shown the morphogen to be required for DA neuron formation, with the null mutants showing a complete loss of ventral progenitor cells (Blaess et al. 2006). Interestingly, conditional knockout of Shh at E9.0 in mouse allows the presence of a small number of TH-positive neurons. When Shh is allowed to persist until E11.5, a full complement of dopaminergic cells develop (Blaess et al. 2006).

Shh mediates its effect through its receptor Patched (Ptc) and a signaling pathway including the Smoothed protein (Smo), which eventually converges on the Gli family of transcription factors (Fuccillo et al. 2006; Fogel et al. 2008). Ectopic expression of either Shh or the Gli1 effector protein in the MHB can induce DA neurons ectopically in the dorsal midbrain (Hynes et al. 1997), and inactivation of Smo at E9 (in mice) results in a large decrease in the numbers of DA neurons (Blaess et al. 2006). These observations further enhance the likelihood that Shh signaling is able to induce a DA phenotype, possibly through regulation of other signalling pathways.

Engrailed

The expression of the two *Engrailed* genes (*Engrailed 1* and *Engrailed 2*; *En1/2*) in the anterior neural folds is initiated during early somite formation at approximately E8.5 in mouse. During later development, expression is maintained by Wnt1 (Danielian and McMahon 1996) where they hybridize in a ring around the neural tube at the point of the MHB. Although the two genes mark the same set of cells, expression differs in intensity at this point, with *En2* showing stronger expression, particularly within the germinal zone (Davis and Joyner 1988).

Mutant mice null for both *En1* and *En2* show morphological defects as early as E9 (Liu and Joyner 2001) and immunolabeling with TH antibody showed a complete loss of the DA neurons normally present within the SNc and VTA (Simon et al. 2001). Ablation of a single *Engrailed* gene, however, leads to an almost exact replication of the wild-type midbrain, thus indicating that the genes can functionally compensate for the loss of each other (Hanks et al. 1995; Simon et al. 2001). Homozygous-heterozygous mutations show differing severities in their phenotype with *En1*^{-/-}; *En2*^{-/+} mutations having a more severe reduction in DA neurons than *En1*^{-/+}; *En2*^{-/-} animals (Simon et al. 2001), possibly indicating that *En1* is a more potent factor than *En2*.

Wnt Proteins

The Wnt family are secreted, glycosylated proteins and have many functions attributed to them, including control of proliferation, differentiation, patterning, cell polarity, axon guidance, and cell death (Lyuksyutova et al. 2003; Hirabayashi et al. 2004; Willert et al. 2003; Prakash and Wurst 2007). Their transcription-activation effects are mediated through the β -catenin (canonical) pathway involving the frizzled receptor and dishevelled signal transducer. Wnt1 is expressed in the caudal midbrain and in two stripes either side of the ventral midline along the mesencephalic flexure in response to the formation of the MHB (Davis and Joyner 1988; McMahon et al. 1992; Parr et al. 1993). The expression domain overlaps with the region that will later give rise to the DA progenitor pool and mature DA neurons. Two distinct phases of DA neuron generation are effected by Wnt1: generation of the DA progenitor pool and terminal differentiation of neurons (Prakash et al. 2006). During the establishment of a competent progenitor cell population, Wnt1 regulates a genetic network including *Otx2* and *Nkx2.2* that is required for the establishment of the DA progenitor pool. Maintaining *Otx2* expression is a key step toward the specification of DA neurons as it functions not only in defining the midbrain territory and establishing the MHB through a repressive interaction with *Gbx2*, but also much later in cell specific patterning events in the ventral neural tube.

A second member of the Wnt family involved in DA development is Wnt5a, which is expressed and secreted by glial cells. Classically, glial cells have been described as supportive scaffold cells, thus aiding the survival of neurons (O'Malley et al. 1992). More recently, however, they have been implicated in further developmental roles (Petrova et al. 2003). To demonstrate the ability of glial cell-derived Wnt5a to aid DA development, a Wnt5a blocking antibody has been used to prevent its functioning in culture. When E14 rat VM precursor cells were cocultured with glial cells in this condition, a reduction of 26% in the number of TH-positive neurons was observed (Castelo-Branco et al. 2006). As with Wnt1, Wnt5a had previously been shown to increase the number of TH-positive cells obtained from E14.5 precursor cells when applied as a partially purified protein; however, the mechanisms by which this is achieved differ: Wnt1 by regulating nuclear receptor related 1 (Nurr1) positive precursor cell proliferation; and Wnt5a by promoting the conversion of Nurr1 positive cells into DA neurons. (Castelo-Branco et al. 2003).

Otx2

In addition to roles in patterning the midbrain territory, *Otx2* also functions in the development of midbrain progenitor

cells. Multiple mutants have been generated to investigate further roles attributable to *Otx2*. In conditional knockouts where *Otx2* is deleted from E9.5 onwards, *Shh* expression expands dorsally and the FGF8 domain moves into to midbrain from anterior hindbrain (Puelles et al. 2004). This tends to indicate that in addition to defining the midbrain territory, *Otx2* may function to preserve this territory and prevent encroachment of hindbrain neuronal phenotypes. It may also serve to prevent expansion of the ventral midbrain territory by preventing *Shh* expression in more dorsal regions. Although a small part of the midbrain tissue is generated in this conditional knockout mutant, the expression of *Nkx2.2* expands ventrally and the expected DA neurons are replaced by those having a serotonergic phenotype. Thus, *Otx2* is required also for preventing *Nkx2.2* expression and hence a serotonergic neural phenotype in the ventral midbrain (Prakash et al. 2006). In a second conditional knockout of *Otx2* at E10.5, the proneural genes *Ngn2* and *Mash1* are not expressed, resulting in a loss of DA neurons at the ventral midline. Again, the expression of *Nkx2.2* expands ventrally (Vernay et al. 2005).

Paired Box Genes Pax2 and Pax5

The expression of the paired box gene *Pax2* begins before the formation of an obvious neural plate. Before *Wnt1* or *En1/2* are observed, the expression becomes restricted to the presumptive MHB region (Rowitch and McMahon 1995). The related *Pax5* and *Pax8* genes are sequentially activated with overlapping expression patterns (Rowitch and McMahon 1995; Urbanek et al. 1994), making the paired box family of genes popular candidates to play a significant role in the development of this region. *Pax2* and *Pax5* are known to cooperate in the development of the MHB (Urbanek et al. 1997; Schwarz et al. 1997a), which is likely to be through the regulation of *En2* (Li Song and Joyner 2000; Bouchard et al. 2005). Additionally, *Pax2* is sufficient and required for the induction of FGF8 expression from the MHB (Ye et al. 2001). In zebrafish, the inhibition of *Pax2* by neutralizing antibodies results in the loss of the midbrain region (Krauss et al. 1992); the highly conserved function of the paired box genes throughout evolution (Schwarz et al. 1997a) would suggest that this may also be the case in vertebrate development.

Cooperation between *Pax2* and *Pax5* is likely to be critical in the correct development of mid/hindbrain regions. Analysis of homozygous and heterozygous null mutants for *Pax2* and *Pax5*, and the compound mutants reveals varying morphological abnormalities (Urbanek et al. 1997; Schwarz et al. 1997b). Deletion of a single *Pax2* allele has no effect on midbrain development. When this mutant is compounded with heterozygous and homozygous inactivation of *Pax5*, an

increasing loss of posterior midbrain territory occurs, with the more severe mutant demonstrating almost a complete loss of tissue originating from the MHB as early as E9.5.

Sox Genes

Following the production of a progenitor pool of competent cells, these must be maintained in a proliferative, pluripotent state. *Sox* genes encode proteins within the high-mobility group (HMG) family that confer a neural progenitor identity. The expression of *Sox* genes of the SOXB1 subfamily (*Sox1*, *Sox2*, and *Sox3*) correlates with ectodermal cells competent to acquire a neuronal fate, followed by the commitment of cells to a neuronal fate (Pevny and Placzek 2005) and is required to maintain a pluripotent state of the precursor cells (Bylund et al. 2003). Indeed, the ability of proneural genes to induce a specific fate requires the ability to suppress the expression of the SOXB1 subfamily in CNS progenitor cells (Bylund et al. 2003). *Sox3* expression has also been associated with FGF signaling (Saarimäki-Vire et al. 2007) and may be the reason why the loss of FGF8 expression results in a loss of presumptive midbrain territory.

Specification of a Midbrain DA Phenotype

For mature DA neurons to arise from a ventralized progenitor pool of cells, significant further steps are required. Many factors have been implicated in this process, and recently, significant progress has been made in the elucidation of these signaling cascades.

Fibroblast Growth Factors

Fibroblast growth factor 8 is expressed in the anterior midbrain in cells of the MHB at E8 in mouse, indicating a role in postgastrulation development of the nervous system (Heikinheimo et al. 1994). Its expression is maintained, but not initiated by, *Wnt1* (Canning et al. 2007; Lee et al. 1997). Functionally, FGF8 serves to maintain the MHB (Canning et al. 2007), and hence may be responsible for the signals emanating from the organizing center. Therefore, the role of FGF8 in DA neural specification may be an indirect one, functioning by maintaining expression of key genes such as *En1/2* and *Pax2*, rather than directly acting upon the cells.

Beads containing the FGF8 protein are able to induce an ectopic isthmus organizer capable of expressing proteins normally associated with this structure such as *En2* and *Wnt1* (Crossley et al. 1996). Since then, it has been shown that FGF8 expression, when overlapping with *Shh* expression from the notochord of the ventral floorplate, creates an

induction site for dopaminergic neurons (Fig.2) (Ye et al. 1998; Roussa and Kriegstein 2004).

Forebrain tissue is usually devoid of dopaminergic neurons; however, ectopic DA cells can be induced in ventral explants by culturing with beads containing the FGF8 protein. Additionally in dorsal tissue, although FGF8 alone is insufficient to induce ectopic expression, DA neurons can be induced when the protein-containing beads are added to explant cultures containing Shh protein. This effect was abolished by the addition of Shh blocking antibodies (Ye et al. 1998).

FGF8 is present as several isoforms (a,b,e and f) due to alternative splicing (Gemel et al. 1996). These differ in their N-termini and biological activity, and have different effects when expressed in the midbrain. Ectopic expression of FGF8a in the midbrain expands the region (Lee et al. 1997); however, FGF8b causes the midbrain territory to undergo transformation into cerebellum (Liu et al. 1999). Other FGF family members shown to play a role in DA development include the neurotrophic FGF20, which has been suggested to play a role in cell survival (Ohmachi et al. 2003). It is expressed throughout the substantia nigra and has been reported to act preferentially on DA neurons (Ohmachi et al. 2000). Coculture of FGF20-overexpressing Schwann cells with Nurr1 positive neural stem cells has been shown to increase differentiation to a TH-positive cell fate (Grothe et al. 2004). Effects of FGF20 have also been observed on human embryonic stem cell differentiation, with cultures containing the growth factor demonstrating a five fold increase in TH-positive neurons; an effect mediated at least in part by a reduction in apoptosis (Correia et al. 2007).

Detection and interpretation of the FGF signals *in vivo* are through the FGF receptors (FGFR) 1, 2, and 3, which are expressed in the developing midbrain (Walshe and Mason 2000; Trokovic et al. 2005). Analysis of mutant mice carrying combinations of *Fgfr1*, *Fgfr2*, and *Fgfr3* mutations reveal that there is a degree of redundancy within the FGF receptor family and that they may be able to functionally compensate for the loss of one another (Saarimäki-Vire et al. 2007).

Transforming Growth Factor Beta

Until recently, members of the TGF- β super-family have been more associated with survival of postmitotic DA neurons, rather than their specification (Unsicker and Kriegstein 2002; Henrich-Noack et al. 1994; Roussa et al. 2004). For example, it has been suggested that GDNF may act as a neurotrophic survival factor for cultured DA neurons (Poulsen et al. 1994). An early expression of TGF- β in the floor plate and underlying notochord of the developing neural tube (Unsicker et al. 1996; Flanders et al. 1991), however, would indicate a much earlier role in the specification of either progenitor cells or terminally differentiated neurons.

Indeed, more recently, TGF- β has been suggested as a critical component of the inductive pathway for DA neurons. The treatment of rat VM cells with TGF- β *in vitro* increases the number of TH-positive cells, while the abolishment of TGF- β prevents TH-positive cell induction by Shh. Additionally, *in vivo* neutralization of TGF- β results in a specific loss of DA neurons (Farkas et al. 2003). This correlates well with the observation that *TGF- β 2* and *TGF- β 3* double knockout mutants show a decreased pool of TH-positive cells within the midbrain (Roussa et al. 2006). Maybe the most conclusive evidence that TGF- β plays a key role during the development of DA neurons is its ability to induce TH-positive neurons in dorsal mesencephalic tissue independent of Shh and FGF8 (Roussa et al. 2006).

The TGF- β pathway also aids DA neuron production by preventing apoptosis during the programmed cell death phase of development through interaction of the homeodomain interacting protein kinase 2 (HIPK2) with Smad2 and TGF- β (Zhang et al. 2007). The expression of HIPK2 is detected in the SNc and VTA regions from E15.5 in mouse and remains high during postnatal development. The midbrains of *Hipk2* null mutants develop normally; however, as early as P0, a substantial reduction in DA neurons is noticeable.

Regulation of Midbrain Development by Transcription Factors

Regulation of a number of transcription factors by Shh signaling mediates the development of the ventral-most cells of the spinal cord (Briscoe et al. 2000). Recently, a number of transcription factors have been identified in the developing midbrain that may indicate this method of development and the specification of cells is replicated throughout the neural tube.

During the development of the midbrain and associated dopaminergic neurons, a large number of regulatory factors are involved at different time points. These are summarized in Table 1.

Lmx1a and Msx1

Lmx1a and Msx1 are two such transcription factors that may regulate DA neuron formation. Shh can induce the expression of both of these transcriptional regulators in naïve explants of presumptive midbrain tissue, but not forebrain or hindbrain tissue (Andersson et al. 2006b). This is a good indication that along the antero-posterior axis, different signals may be activated by Shh signaling. Immunohistochemical investigation reveals expression of Lmx1a and Msx1 in DA progenitor

Table 1 Factors regulating DA neuron generation

Protein	Earliest expression	Function	References
Otx2	E7.0	Midbrain regionalisation, location of MHB and specification of DA phenotype	Broccoli et al. (1999), Millet et al. (1999)
Foxa2	E7.0	Progenitor cell neurogenesis, Nurr1 and En1 expression in immature neurons	Ferri et al. (2007)
Pax2	E7.5	Regulation of En1/2 and induction of FGF8 expression	Li Song and Joyner (2000), Bouchard et al. (2005), Ye et al. (2001)
Lmx1b	E7.5	Maintains Wnt1 expression, terminal DA neuron specification	Adams et al. (2000), Smidt et al. (2000)
Foxa1	E7.5	Progenitor cell neurogenesis, Nurr1 and En1 expression in immature neurons	Ferri et al. (2007)
En1/2	E8.0	DA survival and maintenance	Simon et al. (2001)
Wnt1	E8.0	Regulation of En1/2 expression, DA progenitor cell formation, terminal DA neuron differentiation	Danielian and McMahon (1996), Prakash et al. (2006), McMahon et al. (1992)
Shh	E8.0	Ventral patterning, induction of DA neurons, may regulate transcription factor expression	Hynes et al. (1995), Hynes et al. (1997), Andersson et al. (2006b)
FGF8	E8.0	Maintain MHB, may specify positional information	Canning et al. (2007), Ye et al. (1998), Roussa and Kriegstein (2004)
Lmx1a	E9.0	Control of Msx1 expression, DA neuron specification	Andersson et al. (2006b)
Msx1	E9.0	Control of DA neuron specification, control of Ngn2 expression	Andersson et al. (2006a,b), Kele et al. (2006)
Ngn2	E10	DA specification	Andersson et al. (2007)
Nurr1	E10.5	Terminal DA neuron specification, regulation of TH, AADC, DAT and VMAT	Zetterstrom et al. (1997), Baffi et al. (1999), Backman et al. (1999), Sakurada et al. (1999), Hermanson et al. (2003)
Pitx3	E11.0	Maintenance of SNc neurons	Hwang et al. (2003), Maxwell et al. (2005), Smidt et al. (2004)

Many proteins are required at different stages of midbrain dopaminergic development. This table summarises the key proteins along with their main known functions and onset of expression in mouse

cells immediately above the differentiating neurons (Andersson et al. 2006b). *Msx1* is expressed exclusively in mitotic DA progenitor cells that have not initiated expression of *Nurr1* and TH, whereas *Lmx1b* continues to be expressed in postmitotic *Nurr1*/TH-positive neurons.

Transfection of *Lmx1a* expressing vectors results in an extensive induction of ectopic *Nurr1*/*Lmx1b*/TH-positive neurons. Neural induction was however preceded by the expression of *Msx1*, thus indicating that *Msx1* expression may be controlled by *Lmx1a*. Additionally, the removal of *Lmx1a* by siRNA eliminates *Msx1*-positive progenitor cells and vastly reduces the *Nurr1*/*Lmx1b*-positive pool of cells. *Msx1*^{-/-} mice also show a large decrease in the number of *Nurr1*-positive DA neurons (Andersson et al. 2006b). *Lmx1a* was only able to induce this ectopic DA neuron expression in ventral cells and not in dorsal regions. This may be an indication that other factors are required to cooperate with *Lmx1a* that are expressed exclusively in ventral regions of the midbrain.

Lmx1b

Lmx1b is another LIM homeodomain transcription factor closely related to *Lmx1a* and may also play a role in DA specification. In situ hybridization reveals expression in the

presumptive mesencephalic dopaminergic regions as early as E7.5 and an overlapping expression with *Pitx3*-positive cells in later stages (Smidt et al. 2000). TH expression is initiated in *Lmx1b*^{-/-} mice; however, the mature DA marker *Pitx3* is absent. Analysis of the VTA shows a diminished field of TH-positive cells that become extinct by E16 (Smidt et al. 2000). The presence of TH-positive/*Pitx3*-negative cells in *Lmx1b*^{-/-} mice may suggest a role in terminal specification of DA cells; however, the early expression would suggest a function in earlier specification. It has also been noted that the expression of *Lmx1b* diminishes prior to the complete generation of DA neurons, again suggesting an early role (Andersson et al. 2006b). Within the developing chick embryo, it has been shown that *Lmx1b* is required to maintain the expression of *Wnt1* from the MHB (Adams et al. 2000) and that the orthologs *Lmx1b.1* and *Lmx1b.2* are required for the maintenance of the MHB in zebrafish (O'Hara et al. 2005).

Nurr1

Nurr1 is an orphan nuclear receptor protein widely expressed throughout the adult CNS, especially DA neurons. When probed for *Nurr1* mRNA, 96% of SN and 95% of VTA TH-positive cells were double labeled (Backman et al. 1999).

Developmentally, *Nurr1* expression is not observed until E10.5 in mouse, just a few hours before the onset of TH transcription, indicating a role in the later stages of DA neuron specification. Several proteins have been implicated with regulation of *Nurr1*, including the mitogen-activated protein kinases ERK2 and ERK5, and LIM kinase 5 (Sacchetti et al. 2006). Recently, a convergence of the Wnt and *Nurr1* signaling pathways has also been identified, with β -catenin sharing a functional interaction with *Nurr1* (Kitagawa et al. 2007).

In the absence of *Nurr1*, precursor cells are formed, but degenerate and as a result, *Pitx3* expression is decreased (Saucedo-Cardenas et al. 1998). Therefore, it is probable that developmentally, *Nurr1* functions by regulating proteins responsible for the biosynthesis of DA. Indeed, *Nurr1*^{-/-} mutant mice lack both TH and aromatic acid decarboxylase (AADC) (Zetterstrom et al. 1997; Baffi et al. 1999; Le et al. 1999). Several studies have shown *Nurr1* to regulate the expression of many genes required for the conversion of a DA precursor cell into a mature phenotype including TH, AADC, DA Transporter (DAT), and vesicular monoamine transporter (VMAT) (Sakurada et al. 1999, 2001; Hermanson et al. 2003; Volpicelli et al. 2007).

Nurr1 alone is sufficient to generate TH expressing cells from midbrain progenitors in culture; however, these cells do not express markers of a more mature phenotype such as VMAT and *Pitx3*. Mechanistically, *Nurr1* induces this terminal differentiation by inducing cell cycle arrest (Castro et al. 2001). When the basic Helix–Loop–Helix family member Neurogenin2 was expressed with *Nurr1*, mature markers of DA neurons were seen, indicating a synergistic action (Andersson et al. 2007). More recently, however, differences in the actions of *Nurr1* and the proneural protein Neurogenin2 have been identified between species (Park et al. 2008). In cultures of rat neural precursor cells, *Ngn2* repressed the *Nurr1*-induced generation of TH-positive cells. However, in mouse neural progenitor cell culture, *Ngn2* enhanced the effect of *Nurr1* in generating TH-positive cells. This may be an indication that even in such closely related species, differences in the development of the midbrain may exist.

Neurogenin 2

Neurogenin 2 (*Ngn2*) is a basic Helix–Loop–Helix (bHLH) transcriptional regulator belonging to the proneural family of proteins and has been shown to be an important factor in the specification of the DA neural population (Andersson et al. 2006a, 2007; Kele et al. 2006). The expression of *Ngn2* in the developing midbrain is controlled by *Msx1* (Andersson et al. 2006b) in a spatio-temporal pattern coinciding with the generation of DA neurons (Andersson et al. 2006a; Kele et al. 2006).

Analysis of *Ngn*^{-/-} mice shows a severe or total loss of TH-positive DA neurons at early stages of midbrain development; however, the discrepancy between wild-type and mutant mice decreases over time with respect to both number and distribution of DA neurons (Andersson et al. 2006a). This increase in the number of TH-positive neurons over time has been attributed to a second proneural gene showing similar expression, *Mash1*, which may be able to compensate for the loss of *Ngn2* (Kele et al. 2006). The loss of *Ngn2* does not affect the development of other neural subpopulations of the midbrain such as the red nucleus and oculomotor, indicating that *Ngn2* function is restricted to neurons with a DA phenotype (Andersson et al. 2006a). Studies using neural stem and precursor cells suggest that *Ngn2* functions synergistically with *Nurr1* to produce a mature DA phenotype: the overexpression of *Ngn2* increased neuronal differentiation but not DA formation; *Nurr1* overexpression is sufficient to generate TH-positive cells with immature morphology; the overexpression of both *Nurr1* and *Ngn2* together, however, results in mature TH-positive cells expressing a range of DA markers such as VMAT2 and *Pitx3* (Andersson et al. 2007). This would suggest that *Ngn2* alone is capable of specifying a generic neuronal fate, but requires additional factors to direct subtype-specific differentiation. Additional studies utilizing the overexpression of *Ngn2* in neural precursor cells suggest that it is involved in cell cycle exit and neuronal differentiation (Roybon et al. 2008).

Wnt Proteins

During early stages of midbrain development, Wnt proteins play a role in the specification of midbrain territory and the generation of a pool of progenitor cells. At later stages, Wnt proteins play a further role in midbrain development, directing the terminal differentiation of the progenitor cells toward neurons with a DA phenotype. For example, Wnt8 protein has been shown to have a neuralizing effect in the neural tube by blocking the expression of BMP4 in xenopus (Baker et al. 1999).

Another secreted Wnt protein associated with DA neuron generation is Wnt5a. RNA transcripts are observed in the ventral regions of the neural tube and it has been speculated from the early expression patterns that they play a role in the development of this region (Parr et al. 1993). Glial cells of the ventral midbrain have been reported as expressing Wnt5a and have been shown to promote the conversion of VM progenitor cells into DA neurons. The targeted removal of Wnt5a by blocking antibodies reduced this conversion, thus showing a role in DA neuron differentiation (Castelo-Branco et al. 2006).

Wnt1 is expressed by cells of the MHB and is required for correct midbrain formation. Null mutations of *Wnt1* result in a loss of the majority of the midbrain through apoptotic cell death of the early neuroectoderm, possibly because the expression of *En1/En2* is not maintained in its absence (McMahon et al. 1992).

Foxa1 and Foxa2

In a quest to identify further factors controlling DA development, two members of the forkhead family of transcriptional regulators have been identified owing to their expression in ventral midbrain progenitor cells (Ang et al. 1993). Foxa1 and Foxa2 are required throughout multiple phases of DA neuron development, functioning in early progenitor neurogenesis, the expression of Nurr1 and En1 in immature neurons and finally in control of TH and AADC in mature neurons (Ferri et al. 2007). In mice having a null mutation for *Foxa1* with *Foxa2* being conditionally removed at E10.5, *Ngn2* expression was reduced by over half, thus compromising neuronal identity. In mice showing a single mutation, the expression of *Ngn2* was not effected, indicating that Foxa1 and Foxa2 combine to regulate its expression (Ferri et al. 2007). In later stages of development, the double-mutant mouse had decreased the expression of Nurr1 and En1, highlighting the later developmental roles of Foxa1 and Foxa2. This dependence on Foxa1 and Foxa2 was seen to be dosage dependant, with a higher dose being required for DA differentiation effects compared with that required for earlier progenitor cell effects.

Terminal Differentiation and Maintenance

Final commitment of cells to an DA phenotype is accompanied by the onset of TH expression, the rate limiting enzyme in the biosynthesis of the neurotransmitter DA; a step which fails to occur in cells not expressing Nurr1 (Smidt et al. 2003). The DA phenotype is properly confirmed by the expression of Pitx3, which is required to maintain the SNc population of cells (Hwang et al. 2003; Smidt et al. 2004). In the absence of Pitx3, the specific loss of SNc neurons occurs, with the VTA population remaining untouched.

Engrailed

The expression of the *Engrailed* genes is maintained throughout embryonic developmental stages and into adulthood, as demonstrated by double immunolabeling with the DA-specific marker TH (Simon et al. 2001). This suggests a

further role of Engrailed proteins in maintenance of the dopaminergic neurons. Mice showing a double null mutation for *Engrailed* genes do possess a small pool of TH-positive cells at E11; however, these neurons disappear by E14, and the silencing of *Engrailed* genes by RNAi causes DA neurons to rapidly begin apoptotic cell death (Alberi et al. 2004). As demonstrated by mixing VM cells from the *En* null mutant mice with wild-type cells of the same origin, this requirement for continued *En* expression by DA neurons is cell autonomous (Alberi et al. 2004). This may be an indication that *En1/2* are not actually required for DA development during early specification stages, but only for later maintenance by preventing apoptosis during programmed cell death.

Nurr1

Another protein that continues to be expressed in developed DA neurons is Nurr1 (Zetterstrom et al. 1997). It has been shown to cooperate with Pitx3 to promote terminal differentiation to the DA phenotype (Martinat et al. 2006); however, continued expression suggests a prolonged role in maintaining the DA neurons. One theory is that Nurr1 protects neurons by resisting oxidative stress (Sousa et al. 2007). Alternatively, the neurotrophic factor Brain Derived Neurotrophic Factor (BDNF) has also been shown to be regulated by Nurr1 (Volpicelli et al. 2007). As BDNF has previously been shown to act as a trophic factor for DA neurons and increase their survival in mesencephalic cultures (Hyman et al. 1991), this could explain the requirement for Nurr1 for the survival of DA neurons.

Pitx3

The paired-like homeodomain protein Pitx3 is expressed in all DA neurons of the mammalian CNS and is associated with a mature DA phenotype (Smidt et al. 1997, 2000). Using a mutant mouse with eGFP expressed under the control of the Pitx3 promoter, ontogenetic differences were observed between the two populations of neurons in the SN (Maxwell et al. 2005). In a fate mapping analysis, Pitx3 was expressed before TH in cells located ventrolaterally, whereas cells in dorso-medial locations expressed TH before Pitx3. It is therefore likely that Pitx3 is required for TH expression within the SNc neuronal population, but not those of the VTA. This seems to be confirmed by Pitx3 null (aphakia) mice, which are characterized by a complete absence of SNc DA neurons; however, the A10 neurons of the VTA remain intact (Hwang et al. 2003; Nunes et al. 2003). This specific loss of SNc neurons may be an indication that the molecular makeup of neurons of the SNc and VTA differ (Smidt et al. 2004, 2006). Other proteins

involved in DA neuronal specification and maturation such as AADC, Nurr1, and En1 are still expressed in aphakia mice. Thus Pitx3 is not required for the expression of these genes (Smidt et al. 2004; Nunes et al. 2003). This would suggest that Pitx3 is required only for terminal differentiation or maintenance of TH-positive neurons.

Identifying New Proteins Involved in DA Development

Over the last decade or so, our understanding of midbrain development has increased massively. However, the relative inability to transfer this knowledge to generating large numbers of TH-positive neurons from stem cells in culture indicates that there may be other proteins involved that are as yet unidentified. These may be novel proteins, or existing proteins the functions of which have not yet been identified.

In an attempt to further our knowledge of proteins required for the generation of DA neurons, our laboratory has recently undertaken a proteomics-based investigation of the developing midbrain. Proteins were extracted at four

different developmental time points, before (E11), during (E12), and after (E13, E14) peak DA neurogenesis (Gates et al. 2006). Proteins from different ages were digested with trypsin and peptides labeled using isobaric tag for relative and absolute quantitation (iTRAQ) reagents and analyzed by two-dimensional liquid chromatography (2D-LC) followed by tandem mass spectrometry. The pooled labeled tryptic peptides were first separated using strong cation exchange chromatography (SCX). One-minute fractions were collected for a total of 30 min. Each peptide-containing fraction was further separated by reversed phase (RP) chromatography. Fractions were eluted directly onto a matrix-assisted laser desorption/ionization (MALDI) target plate for tandem mass spectrometric analysis. Using the iTRAQ labels, relative protein expression between the four age groups was quantified (Fig. 3).

This method generated a large list of approximately 3,000 proteins and their expression ratios. The list of proteins identified contained several known to be expressed in the developing midbrain such as β -III tubulin, TH, FGFs, and GAP-43. Additionally, large numbers of proteins involved in signaling mechanisms known to be active during the generation of the midbrain, such as long-term potentiation and

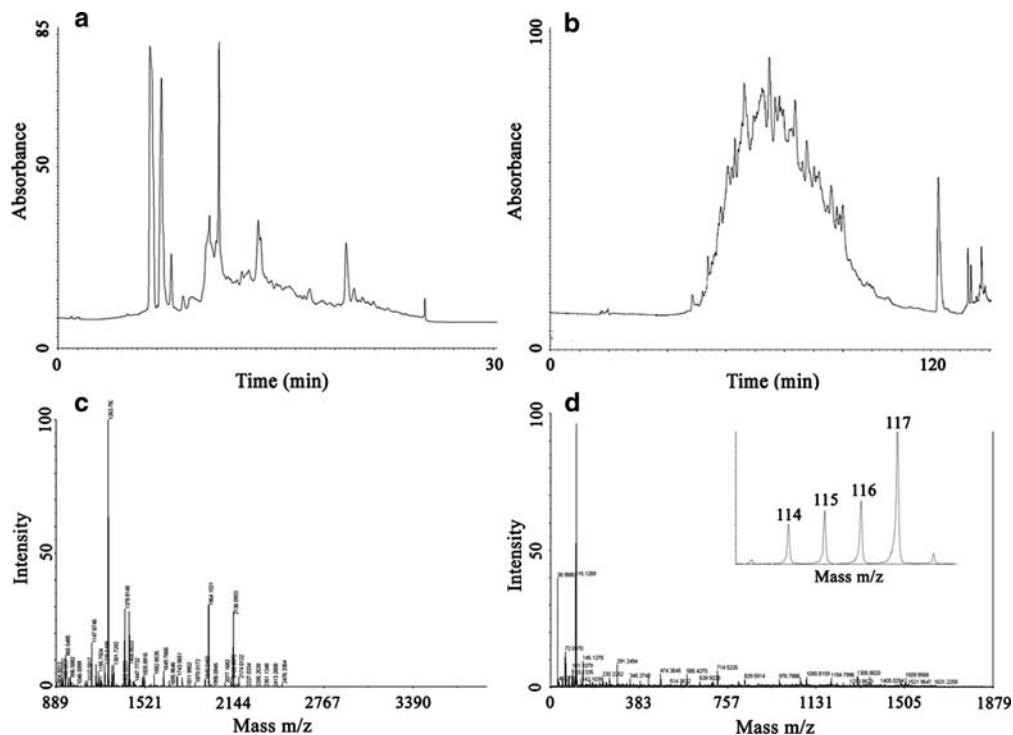
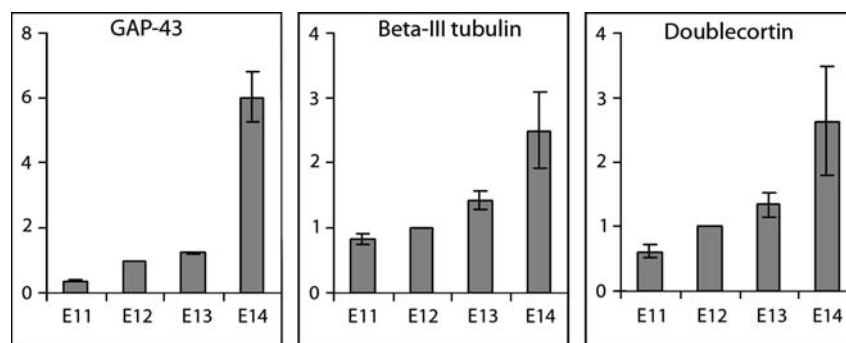


Fig. 3 Protein identification via iTRAQ labelling and proteomics. Protein was extracted from VM tissue, digested with trypsin and labelled with iTRAQ reagents. The combined samples were then separated by SCX chromatography and monitored by dual wavelength UV spectroscopy (a) 1 min fractions were collected for a total of 30 min. Fractions containing peptides as determined by UV absorption were further resolved by RP chromatography using an increasing salt gradient (b) Peptides were automatically spotted onto a MALDI target plate and subjected to tandem mass spectrometric analysis. Intact peptide molecular weight was determined (c) and the most abundant species were then dissociated and fragment ions analysed (d) Relative abundance was determined by from the intensities of the four low molecular weight peaks derived from the dissociated iTRAQ labels (inset D)

Fig. 4 Expression ratios of various proteins known to be expressed in the developing VM. Expression is shown relative to that of E12 and error bars are ± 1 standard deviation. Expression is shown for GAP-43, β -III tubulin and Doublecortin



calcium signaling, were identified. Together, these go some way to validating the protein identification data. To confirm the accuracy of expression profile data across the developmental time points studied, ratios of several proteins were analyzed. Of the known proteins expressed in the area, expression data revealed such trends as would be expected (Fig. 4). GAP-43 (A) is expressed by growing axons, and therefore would be expected to increase throughout the development of the midbrain as axons grow toward their target structures; β -III tubulin (B) is a neuron-specific protein, and hence would be expected to increase its presence as more neurons are born; and Doublecortin (C) is expressed by immature migrating neurons and therefore would also be expected to increase in abundance during this small time period.

Using gene ontology (GO) terms, over 7% of the proteins were predicted to be involved in developmental processes. A further 30% fell into the category of cellular processes, within which developmental processes accounted for almost 20% of proteins. Using this information, along with expression ratios, several proteins that may play an as-yet-undiscovered role in DA development have been uncovered.

One protein currently under investigation is brain lipid binding protein (BLBP), also known as fatty acid binding protein 7. This was identified from VM tissue with $p < 0.01$ from two peptides LTDSQNFDEYMK and ALGVGFATR, corresponding to amino acids 11–22 and 23–31 respectively. This corresponded to approximately 16% coverage of the protein. Over the course of the four developmental time points studied, BLBP showed an approximate six-fold increase: an expression pattern that matches previous reports (Kurtz et al. 1994). BLBP is expressed in radial glia. It has previously been shown to be a direct target of Notch signaling (Anthony et al. 2005), which is known to play a role in development. As radial glial cells have been identified as the source of most neurons throughout the CNS (Anthony et al. 2004), BLBP could play a part in this development. Alternatively, as neuronal migration may take place on radial glial cells (Hatten 1985), BLBP could act as a guidance cue for axon guidance during development (Anthony et al. 2005).

Closing

In the future, it will be essential to continue studying the emergence of the A9 DA cell group to identify factors that might keep these cells alive longer in patients suffering from PD, or to enable scientist to generate DA neurons that would be readily available for use in a cell replacement strategy for the disease. It is likely that the ability to generate stem cell lines useful for such a strategy will involve the establishment of a cocktail of factors that will need to be mixed with a cell population that is able to respond to these signals appropriately. Whether those cells are obtained from primary dissections of the VM at the right stage of development, or the additional production of stem cell lines that have appropriate receptor/signaling systems that are able to respond to proteins that direct differentiation, it will remain essential to continue to pursue the basic understanding of the ontogeny of this important group of cells.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 2

Phenotype, Compartmental Organization and Differential Vulnerability of Nigral Dopaminergic Neurons

Tomás González-Hernández, Domingo Afonso-Oramas, and Ignacio Cruz-Muros

Abstract The degeneration of nigral dopaminergic (DA-) neurons is the histopathologic hallmark of Parkinson's disease (PD), but not all nigral DA-cells show the same susceptibility to degeneration. This starts in DA-cells in the ventrolateral and caudal regions of the substantia nigra (SN) and progresses to DA-cells in the dorsomedial and rostral regions of the SN and the ventral tegmental area, where many neurons remain intact until the final stages of the disease. This fact indicates a relationship between the topographic distribution of midbrain DA-cells and their differential vulnerability, and the possibility that this differential vulnerability is associated with phenotypic differences between different subpopulations of nigral DA-cells. Studies carried out during the last two decades have contributed to establishing the existence of different compartments of nigral DA-cells according to their neurochemical profile, and a possible relationship between the expression of some factors and the relative vulnerability or resistance of DA-cell subpopulations to degeneration. These aspects are reviewed and discussed here.

Keywords Mesolimbic • Neurochemical profile • Neurodegeneration • Nigrostriatal • Parkinson's disease • Substantia nigra

Abbreviations

DA	Dopamine
DA-	Dopaminergic
PD	Parkinson's disease
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SNcv	Caudo-latero-ventral region of the substantia nigra

SNL	Substantia nigra pars lateralis
SNR	Substantia nigra pars reticulata
SNrm	Rostro-medio-dorsal region of the substantia nigra
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area

Introduction

The idea that the DA-neurons of the substantia nigra (SN) do not constitute a homogenous cell population arose 70 years ago, before the discovery of their monoaminergic nature (Falck et al. 1962) and their striatal connections (Moore et al. 1971), when Hassler (1938) reported that the cell loss in Parkinson's disease (PD) preferentially affects the ventrolateral part of the SN, whereas its dorsal part is relatively preserved, suggesting the existence of at least two different subsets of nigral neurons. The use of neuronal tracers in rats and monkeys, at the end of the 1970s and during the 1980s, revealed that DA-cells in the ventral part of the SN project to the dorsal striatum with their terminal field preferentially located in striosomes, while those in the dorsal SN and the neighboring ventral tegmental area (VTA) project to the ventral striatum and the matrix of the dorsal striatum (Gerfen et al. 1987b; Herkenham et al. 1984). This hodological arrangement provided the first morphological support for dividing the mesostriatal system into two components: the nigrostriatal component with high vulnerability to degeneration and the mesolimbic component with low vulnerability to degeneration (Damier et al. 1999b; Fearnley and Lees 1991; German et al. 1989). Thereafter, the use of immunohistochemistry and in situ hybridization has shown differences in the expression pattern of calcium-binding proteins, peptides, transcription factors, and other neuroactive molecules, among different midbrain DA-cell subsets, suggesting a relationship between phenotype and vulnerability. More recently, with the aid of laser capture microdissection, microarray analysis, and real-time PCR, more than 100 genes have been found to be differentially expressed in DA-cells of

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the SN and VTA of rodents (Chung et al. 2005; Greene et al. 2005; Greene 2006). These genes belong to very different categories, including those encoding mitochondrial proteins, proteins involved in metabolism, axon guidance, etc., but the relevance of the differential expression of most of them is still unknown. The aim of this paper is to review the available data about relevant aspects of the phenotypic differences between midbrain DA-cell groups, in particular those supporting the arrangement of nigral DA-cells into different compartments and their significance in differential vulnerability.

Anatomical References and Terminology

Before addressing the specific aspects of this issue, some basic concepts about the anatomical relationship between the nigral DA-cells and other midbrain cell populations, and the terminology used in describing the different midbrain DA-cell groups in the rat, monkey, and human are briefly outlined.

The SN has been divided cytoarchitectural into three different parts: the SN pars compacta (SNC), a horizontal sheet of densely packed medium and large cells that occupies its dorsal third; the SN pars reticulata (SNR), a more diffuse and cell-poor division containing small and medium neurons, lying between the SNC and the cerebral peduncles; and the SN pars lateralis (SNL), a small cluster of medium cells that extends rostrocaudally along the lateral border of the SNC and SNR. The midbrain DA-formation is organized in three cell groups first identified in the rat by Dahlstrom and Fuxe (1964): A8 composed of sparsely arranged DA-neurons in the retrorubral field of the reticular formation; A9 that corresponds to the nigral DA-cells, most of which are localized in the SNC, but also in the SNR and to a lesser extent in the SNL; and A10 composed of several DA-cell nuclei lying in the rostral half of the ventral midbrain, dorsomedial to the SN and ventral to the red nucleus. The term VTA is often used as synonym for A10, because VTA is the largest nucleus of A10. VTA DA-neurons form a continuous body with those in the dorsomedial and rostral region of the SNC, making it impossible to establish the limit between the nuclei by using single TH immunostaining.

Although most relevant features of the A9 cell organization are preserved in rodents, monkeys, and humans, interspecies differences are also evident. For example, the rat SN has been considered as a laminar structure, composed of dorsal, ventral, and ventrally displaced tiers (Gerfen et al. 1987a,b). This arrangement is also evident with the aid of three-dimensional reconstruction. Our studies reveal two densely packed DA-cell bands, one large rostromedial and the other caudoventral, smaller in size, which emits cell-bridges that make contacts with the rostromedial (Gonzalez-Hernandez and

Rodriguez 2000). The rostromedial band corresponds to DA-cells in the SNC, or the dorsal and ventral tiers of Gerfen et al. (1987a,b), and the caudoventral band and cell-bridges to DA-cells in the SNR, or the ventrally displaced tier of Gerfen et al. (1985, 1987a). Although a certain layering may be recognized in monkeys and humans (Olszewski and Baxter 1954), their nigral DA-cells form aggregates in both the dorsal and ventral parts of the SN (McRitchie et al. 1996, 1998). Furthermore, according to Damier et al. (1999a), nigral DA-cells in humans follow a striatum-like arrangement, with several DA-cell clusters (nigrosomes) in the middle of a DA-cell poor field (nigral matrix). These cytoarchitectural differences together with the difficulty in establishing the limit between VTA and SNC have made it difficult to recognize a DA-cell subpopulation through different studies and to identify similarities or differences among different species.

From a practical point of view, bearing in mind the topographical distribution of mesostriatal projections (Fallon and Loughlin 1982; Heimer 2003; Joel and Weiner 2000) and the degeneration pattern in PD (Bernheimer et al. 1973; Damier et al. 1999b; Hirsch et al. 1988) and in animal models of PD (Burns et al. 1983; Chiueh et al. 1985; Gonzalez-Hernandez et al. 2004; Hung and Lee 1996; Rodriguez et al. 2001), nigral DA-cells can also be appropriately divided into two regions: the rostro-medio-dorsal region (SNrm) and the caudo-latero-ventral region (SNcv). In rats, SNrm corresponds to the dorsal tier and the SNcv to the ventral and ventrally displaced tiers (Gerfen et al. 1987a,b; Gonzalez-Hernandez and Rodriguez 2000; McRitchie et al. 1996). Following the nomenclature and parcellation criteria used by different authors, we can say that the SNrm in monkeys and humans corresponds to the ventral half of the dorsal part of the SNC described by Damier et al. (1999a) and Kubis et al. (2000), the dorsal tier of SN described by McRitchie et al. (1996, 1998), the densocellular region of the ventral tier described by Haber et al. (1995), and the β subdivision of the SNC described by Olszewski and Baxter (1954). The SNcv corresponds to the ventral part of the SNC described by Damier et al. (1999a) and Kubis et al. (2000), the ventral tier of the SNC described by McRitchie et al. (1996, 1998), the cell columns of the ventral tier described by Haber et al. (1995), and the α subdivision of the SNC described by Olszewski and Baxter (1954).

Calcium-Binding Proteins in Nigral Neurons

Calcium-binding proteins belong to the superfamily of EF-hand proteins. These proteins are structurally characterized by the presence of a variable number of motives consisting of a helix (E), a loop and another helix (F), which bind Ca^{2+} ions with high-affinity. A number of neuronal actions, from

neurotransmitter release to the activation of transcription factors, are controlled by changes in the cytosolic concentration of Ca^{2+} . These actions may be regulated by two different types of calcium-binding proteins: calcium sensor proteins, which undergo conformational changes on Ca^{2+} binding and consequently interact with specific target proteins thereby modifying their function, and calcium buffer proteins, which modify spatiotemporal aspects of Ca^{2+} but do not undergo conformational changes (Burgoyne and Weiss 2001; Heizmann and Braun 1992; Ikura et al. 2002).

Calbindin- $\text{D}_{28\text{k}}$ (CB), calretinin (CR), and parvalbumin (PV) are three calcium-binding proteins currently considered as calcium buffer proteins that are expressed in different neuronal populations throughout the Central Nervous System (Baimbridge et al. 1992; Rogers et al. 1990), including the SN (Gerfen et al. 1987a; Gonzalez-Hernandez and Rodriguez 2000; McRitchie and Halliday 1995). CB and CR are expressed in DA-cells (Fortin and Parent 1996; Gerfen et al. 1987b; McRitchie et al. 1996), and PV in nigral GABA-cells (Gonzalez-Hernandez and Rodriguez 2000; Reiner and Anderson 1993). CR expressing DA-neurons are abundant in the three midbrain DA-nuclei and in the different nigral regions, although the percentage of double-labeled neurons varies from one study to another (Liang et al. 1996; McRitchie and Halliday 1995; Nemoto et al. 1999). We found CR immunoreactivity in more than 80% of DA-cells in the rat SNC (including dorsal and ventral tiers), SNR, and SNL (Gonzalez-Hernandez and Rodriguez 2000). In contrast, CB is expressed in DA-cells in the VTA, SNrm, and SNL (Gerfen et al. 1987b; Liang et al. 1996; McRitchie and Halliday 1995; McRitchie et al. 1996). Most DA-cells in these regions coexpress CB and CR (Gonzalez-Hernandez and Rodriguez 2000; Nemoto et al. 1999). Interestingly, only DA-cells lying in a disk-shaped region in the lateral half of the rat SNC contain neither CR nor CB (Gonzalez-Hernandez and Rodriguez 2000). The ability of these proteins to buffer intracellular Ca^{2+} , together with the localization of CB in VTA and SNrm DA-cells that are two subpopulations resistant to degeneration, has suggested that the expression of calcium buffer proteins, and in particular CB, confers neuroprotection (Gerfen et al. 1985; German et al. 1992; Yamada et al. 1990). However, despite the fact that most SNrm DA-neurons resistant to DA-neurotoxins express CB, some findings contrast with this hypothesis. For example, no differences have been observed between the neurotoxic effect of MPTP in midbrain-DA cells of CB-deficient mice and their wild-type littermates (Airaksinen et al. 1997). In addition, DA-cells in the SNL that express CB are more sensitive to the neurotoxic effect of 6-OHDA than other nigral DA-cells that do not express CB, such as those in the lateral half of the SNC that express neither CB nor CR (Rodriguez et al. 2001). It is possible that the acute or subacute DA-cell death obtained in animal models of PD (MPTP or 6-OHDA

injection) is not enough to demonstrate the putative neuroprotective effect of calcium-binding proteins in PD. In any case, current data cast doubts about such an effect and suggest that other phenotypic features may be responsible for the resistance of SNrm and VTA DA-cells to degeneration.

Neuropeptides in Nigral DA-Neurons

Neuropeptides are the most abundant chemical mediators in the Nervous System. They are involved in several neuronal and brain functions, acting as either primary neurotransmitters or modulators of classical neurotransmitters (Crawley 1991; Ferraro et al. 2007; Geisler et al. 2006; Wu and Wang 1994). Using in situ hybridization and immunohistochemistry after colchicine injection, two of these, cholecystokinin (CCK) and neurotensin (TN), have been found in midbrain DA-cells, although showing differences in their expression patterns (Fallon et al. 1983; Hokfelt et al. 1980; Palacios et al. 1989; Roubert et al. 2004; Schalling et al. 1990). CCK is expressed in about 70% of DA-cells in the SNrm, most of which are in its anterior half, and in practically 100% of DA-cells in the SNL (Gonzalez-Hernandez and Rodriguez 2000; Seroogy et al. 1989), while NT is only expressed in those neurons lying in the medial portion of the SNC (Seroogy et al. 1988). On the other hand, although midbrain DA-cells do not express the neuropeptide substance P (SP), the fact that the SN receives a dense plexus of SP-immunoreactive striatal afferents has been used to separate SN DA-cells from those in VTA and the retrorubral field, where SP-immunoreactive fibers are very sparse (Gibb 1992; McRitchie and Halliday 1995; McRitchie et al. 1996, 1998).

Dopaminergic Markers in Nigral DA-Neurons

It is currently assumed that DA-cell degeneration, the pathological hallmark of PD, is an oxidative stress-mediated process and that the enzymatic and nonenzymatic catabolism of DA is the main source of reactive oxygen species in DA-cells. Therefore, DA, in addition to being the neurotransmitter the deficit of which characterizes PD, may also be responsible for DA-cell damage, and intercellular differences in the handling of DA may be critical in the differential vulnerability of DA-cells. The cytosolic levels of DA depend on four processes: synthesis, the limiting enzyme of which is tyrosine hydroxylase (TH), packing into synaptic vesicles, release, and reuptake. DA is stored in vesicles by the vesicular monoamine transporter type 2 (VMAT2), a glycoprotein also present in other monoaminergic cells. After its release, DA is taken back by the dopamine transporter (DAT), another glycoprotein only expressed in DA-cells,

the activity of which is regulated by the DA autoreceptors D₂ and D₃. Both the transporters have been proposed as playing opposite roles in DA-cell vulnerability. VMAT2 acts as a neuroprotective factor by sequestering DA into vesicles and preventing its metabolism, and DAT as a vulnerability factor increasing cytosolic DA levels (Miller et al. 1999). In situ hybridization studies carried out during the last two decades reveal internuclear differences in the midbrain expression of different DA-cell markers (Cerruti et al. 1993; Haber et al. 1995; Hurd et al. 1994; Shimada et al. 1992; Uhl et al. 1994; Weiss-Wunder and Chesselet 1991). By using nonradiative riboprobes and PCR analysis in rats, we found the highest mRNA levels for TH, DAT, and VMAT2 in the SNrm, followed by the SNcv, and the lowest ones in A10 (Gonzalez-Hernandez et al. 2004). The fact that dopaminergic markers, including the one with a potential neuroprotective role, display the same expression pattern through the ventral midbrain suggests that their possible involvement in the differential vulnerability of DA-cells could be due to aspects other than differences in their mRNA levels. In this respect, it should be noted that the analysis of protein expression, in contrast to that of their messengers, revealed differences between both transporters. The expression pattern of VMAT2 protein was similar to that described for its messenger, with a close correspondence between VMAT2 mRNA and protein levels in the different midbrain DA-cell subsets. However, the expression pattern of DAT protein was different from that of its messenger, with some DA-cell groups showing high levels of DATmRNA and very low levels of DAT protein, suggesting internuclear differences in the post-translational regulation of DAT. In summary, DAT protein expression in the ventral midbrain of rats, monkeys, and humans follows a caudoventrolateral-to-rostradorsomedial decreasing gradient: SNcv > SNrm > A10. A similar expression pattern was found in their striatal terminal field, with the highest DAT protein levels in the dorsolateral striatum, the target region of SNcv neurons, followed by the dorsomedial striatum, the target region of SNrm neurons, and the lowest DAT protein levels in the ventral striatum, the target region of VTA neurons (Gonzalez-Hernandez et al. 2004). This expression pattern coincides with that of DA-cell degeneration in PD (Damier et al. 1999b; Fearnley and Lees 1991; German et al. 1989; Gibb and Lees 1991; Goto et al. 1989) as well as in monkey (Chiueh et al. 1985; Schneider et al. 1987; Varastet et al. 1994) and rodent (Gonzalez-Hernandez et al. 2004; Rodriguez et al. 2001) models of PD, suggesting that internuclear differences in DAT posttranslational regulation are involved in the differential vulnerability of midbrain DA-neurons.

In this respect, it is known that DAT activity is regulated by the DA autoreceptors D₂ (Cass and Gerhardt 1994; Mayfield and Zahniser 2001; Parsons et al. 1993) and D₃ (Joyce et al. 2004; Zapata et al. 2001). The activation of D₂

autoreceptors inhibits DA-cell firing, DA synthesis, and release, and in contrast to the usual inhibitory role played by autoreceptors, it stimulates DAT activity (Cass and Gerhardt 1994; Parsons et al. 1993; Rothblat and Schneider 1997; Zahniser and Doolen 2001). D₂ receptors have also been considered as being responsible for the neuroprotective effect of DA agonists (Bozzi and Borrelli 2006; Iida et al. 1999; Olanow 1992). However, recent studies show that the DA agonists used in these studies have a much higher affinity for the D₃ type than for the D₂ type receptor (Du et al. 2005; Iravani et al. 2006; Joyce and Millan 2007; Millan et al. 2004), suggesting that D₃ rather than D₂ autoreceptors are primarily involved in neuroprotection. It should be noted that pramipexole, a D₃-receptor preferred agonist, induces a decrease in striatal DA uptake and DAT immunoreactivity in parallel with its neuroprotective effect (Joyce et al. 2004). Hence, it is possible that DAT downregulation may be involved in the neuroprotective effect of D₃ agonists. In spite of these interesting pharmacological findings, anatomical details about D₃ receptor expression in midbrain DA-cells are sparse. In contrast to the D₂ receptor that is strongly expressed and follows a distribution pattern similar to that described for TH and DAT (Haber et al. 1995; Hurd et al. 1994), the D₃ receptor is moderately expressed in midbrain DA-cells and no internuclear differences have been described (Diaz et al. 1995, 2000; Quik et al. 2000). However, the fact that its expression was not significantly reduced in MPTP-treated monkeys has suggested that it is preferentially expressed in a resistant cell subpopulation (Quik et al. 2000). Further studies should be addressed to obtain a more precise anatomical description of its expression pattern in presynaptic cells and to confirm the possible relationship between the neuroprotective effect of D₃ agonists and DAT regulation.

GAD Expression in Nigral DA-Neurons

As mentioned in the introduction, dopaminergic and GABAergic neurons have been considered as being the following two separate and functionally different nigral cell populations: DA-neurons corresponding to the A9 cell group of Dahlstrom and Fuxe (1964), most of which are localized in the SNC and projecting to the striatum (Beckstead et al. 1979; Faull and Mehler 1978); and GABAergic neurons which are localized in the SNR and form one of the most important output pathways of the basal ganglia, projecting to the thalamus, colliculi, and tegmentum (Di Chiara et al. 1979; Grofova et al. 1982; Redgrave et al. 1992). Although this dual arrangement is assumed, several studies had suggested the existence of a nondopaminergic mesostriatal projection (Fibiger et al. 1972; Hattori et al. 1991; van der Kooy et al. 1981), and in 1999 we described a GABAergic nigrostriatal projection

arising from the SNR by using morphological and physiological methods in rats (Rodriguez and Gonzalez-Hernandez 1999). Another possibility is that DA and GABA may be synthesized and function as cotransmitters in nigral cells. In the early 1990s, Campbell and coworkers (1991) described a subpopulation of neurons in the ventrolateral region of the SNR that projected to the superior colliculus, and showed double labeling for TH and glutamic acid decarboxylase (GAD, the rate-limiting enzyme in GABA synthesis). More recently, Hedou et al. (2000) reported that many DA-neurons through the SNC display immunoreactivity for GAD and succinic semialdehyde reductase, the synthesizing enzyme of γ -Hydroxybutyrate, a metabolite of GABA with neurotransmitter and neuromodulator properties. By using in situ hybridization for the two GAD isoforms (GAD65 and GAD67), TH immunohistochemistry and fluoro-gold injection in the striatum, we also found that a subpopulation of mesostriatal DA-cells, approximately 10%, most of which are localized in the SNrm and VTA, contain GAD65mRNA but not GAD67mRNA (Gonzalez-Hernandez et al. 2001), in contrast to genuine GABAergic neurons that express both GAD isoforms (Esclapez et al. 1993, 1994; Mercugliano et al. 1992). It is known that while GAD76 is widely distributed throughout the neuron as an active holoenzyme form, associated with functions requiring high levels of GABA synthesis, GAD65 localizes in terminals as an inactive apoenzyme form, providing a reservoir of GAD, which is regulated by energy metabolites (Kaufman et al. 1991; Martin et al. 1991; Meeley and Martin 1983; Miller et al. 1978; Spink et al. 1985). This can explain why we failed to find GAD and GABA immunoreactivity in midbrain DA-cell somata, and suggests that DA-cells expressing GAD65mRNA would synthesize GABA at terminal levels in response to local demands. Although GABA receptors have been found in different striatal cell types as well as in glutamatergic and dopaminergic terminals (Ikarashi et al. 1999; Rahman and McBride 2002; Seabrook et al. 1991; Smolders et al. 1995), the fact that striatal DA-terminals contain GABA receptors (Doherty and Gratton 2007; Ronken et al. 1993), and that a subpopulation of DA-cells in the SNrm and VTA contain GADmRNA, suggest that GABA released from these cell subpopulation can exert a short auto-regulatory mechanism of DA release in the mesolimbic system.

Expression of Glutamate Receptors in the Substantia Nigra

Glutamate is the major excitatory neurotransmitter in the mammalian brain, and also a potent neurotoxin when it reaches high extracellular concentrations. Glutamatergic actions are exerted through the activation of two families of

glutamate receptors: ionotropic, which form ion channels; and metabotropic, which are coupled by G-proteins to different second messenger systems. According to their agonist selectivity, ionotropic receptors have been classified into three groups: N-Methyl-D-Aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), and kainic acid (KA), all of which are composed of different subunits and variants, which determine their biological actions (Burnashev et al. 1992; Geiger et al. 1995; Hollmann and Heinemann 1994). Metabotropic receptors (mGluRs) have also been divided into three groups: Group I, which comprises mGluR1 and mGluR5; Group II, which comprises mGluR2 and mGluR3; and Group III, which comprises mGluR4, mGluR6, mGluR7, and mGluR8 (Conn and Pin 1997). Studies performed during the last decade indicate that KA receptors also act by means of G-protein-dependent intracellular signaling cascades (Rodriguez-Moreno and Sihra 2007).

Nigral DA-cells receive glutamatergic afferents from the subthalamic nucleus, cerebral cortex, and pedunculopontine tegmental nucleus (Charara et al. 1996; Forster and Blaha 2003; Iribe et al. 1999; Smith et al. 1990). These projections play a modulatory role on the basal activity of DA-cells (Grace and Bunney 1984; Smith and Grace 1992), and also contribute to DA-cell degeneration, mostly by means of the hyperactivity of the subthalamic nucleus (DeLong 1990; Rodriguez et al. 1998). It is known that ionotropic receptors, particularly the NMDA group, play a fundamental role in this effect (Nash et al. 1999; Konitsiotis et al. 2000; Sonsalla et al. 1998), but an increasing body of evidence supports the involvement of other glutamate receptor types (Armentero et al. 2006; Bonsi et al. 2007; Vernon et al. 2007). Consequently, the expression pattern and subunit composition of glutamate receptors in midbrain DA-cells may contribute to their differential susceptibility to degeneration.

By using immunohistochemistry and in situ hybridization in the study of NMDA and AMPA receptor expression in the monkey midbrain, Paquet and coworkers (1997) found that the expression of the subunits NMDAR1 and Glu2R of the NMDA and AMPA receptors respectively is higher in DA-cells neurons of the ventral tier of the SN than in those of its dorsal tier and VTA, suggesting an association between them as well as a vulnerability. However, the analysis of different subunits of the NMDA receptor (NMDAR1, NMDAR2A-D) in the midbrain of postmortem humans revealed a different expression pattern (Counihan et al. 1998). The highest levels of NMDAR1 and NMDAR2D mRNAs were found in the lateral part of the SN rather than in the ventral tier. This finding suggested the lack of a relationship between the expression of NMDA receptors and vulnerability, because of the assumption that DA-cells in the ventral tier are more vulnerable than those in the lateral region of the SN. This idea should be reconsidered in the light of the pattern of DA-cell loss described by

Damier and coworkers (1999b) in Parkinsonian patients, according to which the highest DA-cell loss is in “nigrosome 1,” localized in the caudolateral region of the ventral tier. However, in the same study, DA-cells in the paranigral nucleus showed higher levels of NMDAR1 and NMDAR2D mRNAs than those in both SN tiers. Bearing in mind that the paranigral nucleus belongs to the A10 group, occupying a dorsomedial position, and that its neurons are relatively preserved in PD, the expression of NMDA receptors itself does not seem to be a determinant of vulnerability.

KA receptors are composed of combinations of 5 different subunits: GluR5, GluR6, GluR7, KA1, and KA2 (Bettler and Mülle 1995; Chittajallu et al. 1999). They are present in GABAergic nerve terminals projecting to nigral DA-neurons, where their activation potentiates spontaneous inhibitory synaptic transmission contributing to the fine control of excitability and firing pattern of DA-cells (Kerchner et al. 2001; Nakamura et al. 2003). The expression of different KA receptor subunits has been reported in midbrain DA-neurons of rodents using in situ hybridization, although current data are sparse and inconsistent. According to Bischoff et al. (1997), DA-cells in the mouse VTA and SN express GluR7, but only those in the SN express GluR5. According to Wullner et al. (1997), nigral DA-cells in rats express KA2 but not GluR5.

Metabotropic glutamate receptors have also been involved in DA release and in the vulnerability of midbrain DA-cells (Bonsi et al. 2007; Golembiowska et al. 2002; Vernon et al. 2007). Group I receptors stimulate and group II/III receptors inhibit DA release by acting either directly on DA-cells or indirectly via the suppression of glutamate release from subthalamic or cortical terminals (Pisani et al. 1997, 2000; Wigmore and Lacey 1998). Moreover, group I receptor antagonists and group II/III receptor agonists ameliorate motor symptoms and protect DA-cells in different experimental models of PD (Battaglia et al. 2003; Dawson et al. 2000). Metabotropic glutamate receptors are expressed at the presynaptic and postsynaptic level in different elements of the nigrostriatal and striatonigral pathways (Berthele et al. 1998; Martin et al. 1992; Smith et al. 2001). Studies focused on their expression in the SN indicate that mGluR1, mGluR5, and mGluR3 are expressed in the SNR and that mGluR1 and to a lesser degree mGluR5 are expressed in DA-cells of the SNC (Hubert et al. 2001; Kosinski et al. 1998).

Nitric Oxide Synthase Expression in the Substantia Nigra

Nitric oxide (NO) is a free-radical gas involved in a wide range of physiological functions and pathological processes (Guix et al. 2005). NO is synthesized by the enzyme nitric

oxide synthase (NOS) in the reaction where the amino acid L-arginine is oxidized to L-citrulline and NO is formed as a by-product. Three different NOS forms have been described, all requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen: neuronal NOS (nNOS or NOS I), endothelial NOS (eNOS or NOS III), and inducible NOS (iNOS or NOS II). nNOS is constitutively expressed in distinct neuronal populations (Bredt and Snyder 1994; Gonzalez-Hernandez et al. 1996), and in other cell types viz., astrocytes, skeletal and cardiac myocytes, and lung epithelial cells (Asano et al. 1994; Kobzik et al. 1994; Xu et al. 1999). Various splice variants of the human nNOS gene have been identified. One of them is the mitochondrial NOS form (Eliasson et al. 1997; Hall et al. 1994; Tatoyan and Giulivi 1998). eNOS is constitutively expressed in the endothelium (Marsden et al. 1993) and also in other cells, viz., neurons and astrocytes (Colasanti et al. 1998). iNOS is induced in response to inflammatory stimuli in immune and glial cells (Galea et al. 1998) and has also been found in hepatocytes and sinusoidal, endothelial, and smooth vascular cells (Kanno et al. 1993; Mohammed et al. 2003). nNOS and eNOS activities are posttranslationally regulated by the phosphorylation and the binding of Ca^{2+} -calmodulin. They usually generate small amounts of NO, which play signaling or effector roles in diverse physiological processes such as vasodilation, neurotransmission, and immune response (Bredt and Snyder 1994; Garthwaite 1991). However, the excitotoxic activation of NMDA receptors can induce nNOS dephosphorylation, which leads to the production of toxic levels of NO and cell death by different mechanisms (Rameau et al. 2003, 2007; Singh and Dikshit 2007). iNOS is Ca^{2+} independent, preferentially regulated at transcriptional level and generates large amounts of NO, which are potentially neurotoxic (Guix et al. 2005; Iadecola et al. 1995).

Data arising from different studies suggest that NO is involved in the pathogenesis of PD. For example, high levels of NOS expression have been found in the SN of PD patients (Hunot et al. 1996) and a MPTP mouse model of PD (Muramatsu et al. 2003). Polymorphonuclear cells from PD patients show an increase of NO production, overexpression of nNOS, and accumulation of nitrotyrosine-containing proteins (Gatto et al. 2000). Genetic deficiency in nNOS or iNOS and treatment with NO-inhibitors protect against DA-cell degeneration induced by MPTP (Liberatore et al. 1999; Przedborski et al. 1996; Schulz et al. 1995), and minocycline, a tetracycline derivative, inhibits iNOS activity and reduces the neurotoxic effect of MPTP (Du et al. 2001). Thus, both nNOS and iNOS seem to be involved in DA-cell degeneration, and glial and/or recruited inflammatory cells could be sources of NO. However, the possible neuronal origin of NO in this process has not been clarified. NOS expression in the SN is very weak under normal conditions.

It is practically restricted to a subpopulation of GABAergic neurons in the lateral region of the SNR (Gonzalez-Hernandez and Rodriguez 2000), and with the exception of a very few DA-cells in the rostromedial region of the SNC, midbrain DA-cells do not express NOS (Bredt et al. 1991; Gonzalez-Hernandez and Rodriguez 2000). An important nitrinergic, together with glutamatergic and cholinergic, input to the SN comes from the pedunculopontine tegmental nucleus (PPN) (Dun et al. 1994; Geula et al. 1993), the neurons of which also degenerate in PD (Hirsch et al. 1987). Studies conducted in our laboratory showed that after an excitotoxic lesion of the PPN, many nigral neurons die without an evident increase of NOS activity in the SN. However, after a non-excitotoxic lesion of the PPN, which produces the loss of most cholinergic–nitrinergic neurons, only a few nigral neurons degenerate, but many nigral neurons become intensely immunoreactive for nNOS (Gonzalez-Hernandez et al. 1997). Although the dopaminergic or GABAergic phenotype of these neurons was not established, the finding suggests that the excitotoxic damage is not mediated by NOS activity in this paradigm and that the induction of nNOS in the SN may be a compensatory mechanism for maintaining local NO levels.

Neurotrophic Factors and Their Receptors in Nigral DA-Neurons

Neurotrophic factors are diffusible peptides that support development, differentiation, and maintenance of specific neuronal populations. They are usually secreted by target cells and act via retrograde signaling by an autocrine or paracrine mechanism, interacting with multicomponent receptor complexes. Most of them have been grouped into different families of structurally and functionally related molecules: nerve growth factor (NGF) superfamily, glial cell line-derived neurotrophic (GDNF) family, neurokinin superfamily, and nonneuronal growth factor superfamily. Members of the different neurotrophic factor families have been shown to exert neurotrophic actions on DA-cells during development, and some of them a neuroprotective effect in “*in vitro*” and animal models of PD (Bradford et al. 1999). The potential use of neurotrophic factors in the design of new therapeutic strategies for PD has prompted neuroscientists to make an effort to better understand their ligand-receptor interactions, regulation mechanisms, and downstream signaling in the mesostriatal system. Understanding the normal expression pattern of neurotrophic factors and their receptors is critical to understanding their effects after being exogenously administered. Morphological studies carried out since the beginning of 1990s have provided interesting data on the expression of neurotrophic factors and their receptors in

the midbrain dopaminergic formation, but numerous aspects are still unknown or controversial.

The GDNF family is a group of particular interest in midbrain DA-cells. It consists of four distant members of the transforming growth factor β superfamily: GDNF, neurturin, artemin, and persephin (Airaksinen et al. 1999). Their biological actions are exerted by means of a receptor complex composed of a common tyrosine kinase subunit (Ret) that acts as the signaling component, and a specific high-affinity ligand-binding protein, the glycosylphosphatidylinositol-anchored GDNF receptor α component. GDNF preferentially binds to GFR α 1, neurturin to GFR α 2, artemin to GFR α 3, and persephin to GFR α 4, although these binding specificities are not exclusive (Baloh et al. 1997; Jing et al. 1996). Since its discovery (Lin et al. 1993), GDNF has attracted substantial attention because it displays a higher degree of neuroprotection and rescue of DA-cells than other neurotrophic factors in both “*in vitro*” and animal models of PD (Bourque and Trudeau 2000; Kordower et al. 2000). Based on these findings, clinical trials have been performed to check its therapeutic value in Parkinsonian patients. However, the results have not been as satisfactory as was expected (Patel and Gill 2007), suggesting that different issues need to be addressed before its clinical use can be widely adopted. In 1996, Neurturin was purified and identified as a member of the GDNF family supporting sympathetic and sensory neurons. (Kotzbauer et al. 1996). Thereafter, neurturin has been shown to support embryonic DA-cells and to protect mature DA-cells from the neurotoxic effect of 6-OHDA to almost the same degree as GDNF, but without inducing sprouting and an increase of TH immunostaining in rescued cell bodies (Akerud et al. 1999; Horger et al. 1998; Li et al. 2003). In addition, striatal delivery of viral vector encoding human neurturin in Parkinsonian monkeys has shown a significant improvement of MPTP-induced motor impairment and midbrain DA-cell degeneration, with a safety and tolerance profile that supports an ongoing clinical program in PD (Gasmi et al. 2007; Kordower et al. 2006).

The advances in the therapeutic use of GDNF and neurturin depend on factors such as a better selection of patients, improvement in delivery methods, monitoring ligand release, and also the knowledge of basic aspects of their action mechanisms, including ligand-receptor interactions, intracellular signaling, and their expression pattern under both normal conditions and in Parkinsonian patients. It is known that both the subunits of the GDNF receptor complex, GFR α -1 and Ret, are robustly expressed in SN and VTA DA-neurons, and that GABA-ergic neurons in the SNR also express GFR α -1, suggesting a different action mechanism in these cells (Barroso-Chinea et al. 2005; Golden et al. 1998; Sarabi et al. 2001; Trupp et al. 1997). The expression of GFR α -2 in the ventral midbrain is weaker than that of

GFR α -1 and Ret, and it appears to be localized in DA-neurons in the SNC and neighboring non DA-neurons (Golden et al. 1998; Horger et al. 1998). The data regarding the expression of their ligands are controversial. For example, in the case of neurturin, some authors found its expression in the striatum but not in the midbrain (Golden et al. 1998), and others in both the striatum and the midbrain (Cho et al. 2004). In the case of GDNF, it is generally assumed that, although it was first identified in a glial cell line (Lin et al. 1993), GDNF is expressed "*in vivo*" in neurons but not in astrocytes (Bizon et al. 1999; Pochon et al. 1997; Trupp et al. 1997). However, as mentioned with neurturin, there is no consensus about which neurons (target and/or afferent neurons) express GDNF. Some studies show GDNFmRNA expression in both striatal neurons and midbrain DA-neurons (Golden et al. 1998; Pochon et al. 1997), while others only found GDNFmRNA in striatal neurons (Trupp et al. 1997). By using *in situ* hybridization and PCR in adult rats, we found GDNFmRNA expression in the striatum but not in the midbrain (Barroso-Chinea et al. 2005). In addition, GDNFmRNA levels in the ventral striatum were higher than in the dorsal striatum. In spite of the fact that GDNFmRNA was not detected in the ventral midbrain, DA-neurons in the VTA and SNrm, but not those in the SNCv, were immunoreactive for GDNF. This immunoreactivity disappeared after colchicine injection, suggesting that GDNF in DA-cell somata is retrogradely transported from the striatum. As DA-neurons in the VTA and SNrm are more resistant than those in the SNCv, we can suggest that the fact that they project to the ventral striatum, where GDNF expression is higher than in the dorsal striatum, is a neuroprotective factor.

With respect to the NGF superfamily, three of its members, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 have been shown to promote the survival of embryonic DA-cells, to increase DA turnover and uptake, and to exert a neuroprotective action in different models of PD (Altar et al. 1994; Hyman et al. 1991; Levivier et al. 1995; Lingor et al. 2000). NGFs function by interacting with the high-affinity tyrosine kinase (Trk) family of transmembrane receptors (Barbacid 1994; Huang and Reichardt 2003; Hubert et al. 2001). BDNF and NT-4/5 are the preferred ligands for TrkB, and NT-3 for TrkC (Kaplan and Miller 2000; Lamballe et al. 1991). *In situ* hybridization studies show that TrkB and TrkC are expressed in all mesencephalic dopaminergic groups (Numan and Seroogy 1999); however, their ligands, BDNF and NT-3, are restrictively expressed in 25–50% of DA-cells in VTA and 10–30% DA-cells in the SN; these are preferentially localized in its dorsomedial region (Seroogy et al. 1994).

Two members of the nonneuronal growth factor family, the basic fibroblast growth factor (bFGF), also called FGF-2, and the insulin-like growth factor-1 (IGF-1) have been

particularly involved in the development, maintenance, and neuroprotection of DA-neurons (Offen et al. 2001; Shavali et al. 2003; Timmer et al. 2007). As regards bFGF, some studies have reported that it is homogeneously expressed in practically all midbrain DA-cells nuclei (Lolova and Lolov 1995; Tooyama et al. 1993, 1994). Others found bFGF expression in both DA-neurons and astrocytes (Chadi et al. 1993; Cintra et al. 1991), in nigral DA- and non DA-neurons, and astrocytes (Bean et al. 1991), or only in astrocytes (Flores et al. 1998). Bearing in mind that at least one form of its receptor, FGFR-1, is expressed in DA-cells (Walker et al. 1998), these discrepancies make it difficult to establish whether paracrine and/or autocrine mechanisms are responsible for bFGF effects on DA cells.

In the case of IFG-1, its receptor, IFG-1R, has been found in glial cells and all midbrain DA-neurons (Quesada et al. 2007). Bearing in mind that the neuroprotective effect of estrogens may be mediated by IGF-1R (Quesada and Micevych 2004; Quesada et al. 2008), it would be interesting to know which cells coexpress estrogen receptors and IGF-1R. The estrogen receptor- α (ER α) is absent or weakly expressed in midbrain DA-cells, but ER β is conspicuously expressed in midbrain DA-neurons and glial cells (Mitra et al. 2003; Quesada et al. 2007). ER β -immunoreactive DA-neurons are preferentially localized in the VTA, where practically all DA-neurons coexpress IGF-1R and ER β , while in the SN only 40% of DA-cells express both receptors (Creutz and Kritzer 2004; Kritzer 1997; Quesada et al. 2007), although their regional distribution has not been described.

In sum, taking into account the expression patterns of the neurotrophic factors and their receptors in the mesostriatal system, their effects in the differential vulnerability of midbrain DA-neurons might be exerted in different ways: by restricted expression of the ligand in a DA-cell subpopulation, as could occur with BDNF and NT-3, suggesting an autocrine effect on a selective cell population; by coexpression of two interacting receptors in a restricted cell population, as may occur with IFG-1R and ER β , and also by differences in the expression levels of the ligand in the target nucleus, without differences in the expression pattern of its receptors, as proposed for GDNF.

GIRK2 in Nigral DA-Neurons

G-protein inward rectifier potassium (GIRK) channels are a subfamily of the inward rectifier K⁺ channels. They are composed of four GIRK subunits (GIRK1-4) arranged as heterotetramers or homotetramers (Breitwieser 2005; Mark and Herlitze 2000). Initial studies developed in the vagus system revealed that GIRK channel activation led to a

hyperpolarization of the membrane potential mediated by an increase of K^+ efflux across the membrane (Del Castillo and Katz 1955; Hutter and Trautwein 1955; Loewi 1921). Current data suggest that neuronal GIRK channels mediate the inhibitory action of different neurotransmitters, including GABA and DA (Brown and Birnbaumer 1990; Hille 1992; Innis and Aghajanian 1987; Lacey et al. 1988). Midbrain DA-cells contain GIRK channels, which are composed of only GIRK2 subunits (Inanobe et al. 1999). In situ hybridization and immunohistochemistry show internuclear differences in its expression pattern. DA-cells in the SNC show a robust GIRK2 expression, DA-cells in the SNL also express GIRK2, but their labeling intensity is weaker than in the SNC, and only a few DA-cells in the VTA express GIRK2 (Murer et al. 1997; Schein et al. 1998). On the basis of this expression pattern, GIRK2 has been used as a marker of nigral DA-cells, together with CB as a marker of VTA DA-cells, in the analysis of fetal DA-cell transplants in animals (Thompson et al. 2005) and Parkinsonian patients (Mendez et al. 2005, 2008), although its relevance in DA-cell vulnerability is still unknown. The facts that the stimulation of D_2 DA-receptors activates GIRK2 channels (Lacey et al. 1987, 1988), that GIRK2 activation hyperpolarizes DA cells (Lacey 1993), and that hyperpolarization increases DA uptake (Sonders et al. 1997) suggest that the inhibitory effects as well as the increase in DA uptake induced by D_2 autoreceptors (Cass and Gerhardt 1994; Mayfield and Zahniser 2001) may be mediated by GIRK2. In contrast to D_2 , D_3 autoreceptors do not interact with GIRK2 (Davila et al. 2003) and their stimulation inhibits DAT activity (Joyce et al. 2004). Hence, differences between D_2 and D_3 effects on DAT regulation could be related to their capability of interacting with GIRK2. However, other data contrast with the idea of a GIRK2-mediated regulation of DAT activity and its possible implication in DA-cell vulnerability. For example, SNL DA-cells, which contain low levels of GIRK2, show high sensitivity to the DAT-dependent-neurotoxins MPTP and 6-OHDA (Rodriguez et al. 2001; Varastet et al. 1994) and GIRK2-immunoreactive DA-cells survive for a long-time after being transplanted (Mendez et al. 2005).

Development of Midbrain DA-Neurons and Phenotype Differences

The main hallmarks of a neuronal group, viz., anatomical position, connections, and chemical profile, are regulated by a sequence of intercellular and intracellular signaling processes occurring during the first stages of development. Although an exhaustive description of the events and factors involved in the development of midbrain DA-cells exceeds

the aim of this review, some aspects deserve mention. The developmental program of midbrain DA-cells may be divided into two different phases: the first directed at defining the molecular and anatomical borders of the midbrain before DA-cells may be distinguished, and the second directed at defining DA-cell phenotype and connections. The midbrain organization depends on the correct specification of the isthmus, a critical region that separates the hindbrain from the forebrain. *Otx* genes, and in particular *Otx2*, are essential in defining the isthmus and the appearance of the midbrain and forebrain. They are restrictively expressed at the midbrain–hindbrain border (Acampora et al. 1995; Matsuo et al. 1995), inducing the expression of important downstream genes (Rhinn et al. 1998). It is also known that the dorsal and ventral midbrain regions follow different developmental cascades. The signaling molecule sonic hedgehog plays a pivotal role in the development of the ventral region, even inducing DA-cell differentiation when signaling together with *Fgf8* (Ye et al. 1998). Other transcription factors such as *En1*, *En2*, *Pax 2*, *Pax 5*, *Wnt1*, and *Lmx1b* are also expressed in the ventral midbrain before DA-cell markers (Smidt et al. 2000; Wassef and Joyner 1997). Recent studies have been particularly focused on the role of *Nurr1* and *Pitx3* in the differentiation and maintenance of midbrain DA-cells. *Nurr1* is an orphan member of the nuclear hormone receptor family of transcription factors, which is expressed in different brain regions (Law et al. 1992; Smidt et al. 1997). Its expression in midbrain DA-cell precursors starts just before that of TH and is maintained until adulthood (Saucedo-Cardenas et al. 1998). Although *Nurr1*-deficient mice die soon after birth, the study of these animals at the perinatal stage reveals that midbrain neurons maintain the expression of the transcription factors *Lmx1b* and *Pitx3*, but not that of TH, DAT, and VMAT2 (Castillo et al. 1998; Smidt et al. 2003; Zetterstrom et al. 1997). This indicates that *Nurr1* is required for the phenotypic differentiation of DA-cells, but not for the induction of other transcription factors.

The homeobox gene *Pitx3* is expressed in three different tissues during development: eye lens, skeletal muscle, and midbrain DA-cells. In contrast to what occurs in the eye and muscle, *Pitx3* expression is maintained in midbrain DA-cells until adulthood. The fact that midbrain DA-neurons are the only ones expressing *Pitx3* and that the expression starts when the neurons reach the ventral position suggests that *Pitx3* is involved in their final differentiation or maintenance, rather than in their proliferation and migration (Smidt et al. 2000). However, there is a controversy about whether *Pitx3* is expressed in all midbrain DA-cells. While some studies report a complete coincidence between *Pitx3* and TH expression in the midbrain (Smidt et al. 1997, 2000, 2004a,b; Zhao et al. 2004), others propose that *Pitx3* is expressed only in a subset of DA-cells (van den Munckhof

et al. 2003), or that Pitx3 mRNA levels differ from one DA-cell subset to another (Korotkova et al. 2005). According to Van den Munkhof et al. (2003), Pitx3 is expressed in most DA-cells in the ventral tier of the SN, but in only a few in the dorsal tier of the SN, and in about 50% in the VTA. In addition, studies in Pitx3-deficient aphakia mice show a severe DA-cell depletion in the ventral tier of the SN, while those in the dorsal tier of the SN and VTA are relatively preserved (Nunes et al. 2003; Smidt et al. 2004a,b; van den Munkhof et al. 2003), suggesting the existence of two DA-cell populations: one Pitx3-dependent in the ventral tier of SN and another Pitx3-independent in the dorsal tier of SN and VTA. The similarity between the distribution pattern of midbrain DA-cells in Pitx3-deficient aphakia mice (Smidt et al. 2004a,b; van den Munkhof et al. 2003) and that observed in their wild-type receiving MPTP (German et al. 1992) and in Parkinsonian patients (Damier et al. 1999b; Fearnley and Lees 1991) suggests that Pitx3-dependent neurons are more sensitive to degeneration than the Pitx3-independent ones. Hence, Pitx3 could be involved in the differential vulnerability of midbrain DA-cells, either by inducing and maintaining vulnerability factors or by repressing those providing neuroprotection.

Concluding Remarks

The concept of nigral DA-cells has changed a lot since their first identification as monoaminergic or dopaminergic neurons (Dahlstrom and Fuxe 1964; Falck et al. 1962). Morphological and molecular studies carried out during the two last decades support the existence of different midbrain DA-cell subpopulations according to their neurochemical profile, and suggest a correlation between topographical distribution, phenotype, and vulnerability. In addition, new differential factors are continuously added to their phenotype, leading to a more complex profile of the distinct DA-cell subsets. In spite of these advances, the functional significance and the precise expression pattern of many factors are still unknown. In the light of current data, further studies should be directed at elucidating the role of these factors in the handling of DA, and the specific weight of each in the differential vulnerability of DA-cells on the one hand, and at establishing an accurate correlation between the compartmental organization and neurochemical profile of nigral DA-cells in humans and in experimental animals on the other. Our efforts in these issues can contribute to a better understanding of the neurobiology of the DA-mesostriatal system and the pathophysiology of PD.

Conflicts of interest statement We declare that we have no conflict of interest before references in all chapters

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Chapter 3

Specific Vulnerability of Substantia Nigra Compacta Neurons

Marten P. Smidt

Abstract The specific loss of substantia nigra compacta (SNc) neurons in Parkinson's disease (PD) has been the main driving force in initiating research efforts to unravel the apparent SNc-specific vulnerability. Initially, metabolic constraints due to high dopamine turnover have been the main focus in the attempts to solve this issue. Recently, it has become clear that fundamental differences in the molecular signature are adding to the neuronal vulnerability and provide specific molecular dependencies. Here, the different processes that define the molecular background of SNc vulnerability are summarized.

Keywords Dopamine • Mesodiencephalon • Midbrain • Parkinson • Vulnerability

Abbreviations

CRE	Cyclization recombinase
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DOPAL	3,4-dihydroxyphenylacetaldehyde
En1/2	Engrailed
GDNF	Glial cell line-derived neurotrophic factor
MAO-A	Monoamine oxidase A
mdDA	Mesodiencephalic dopaminergic
PD	Parkinson's disease
RA	Retinoic acid
Retinol	Vitamin A
RR	Retrorubral
SNc	Substantia nigra compacta
Tgf- β	Transforming growth factor β

VTA	Ventral tegmental area
Wv	Weaver

Introduction

The main group of dopaminergic neurons that is vulnerable in Parkinson's disease (PD) is anatomically identified in the human brain by the presence of a dark pigment, and hence called the substantia nigra compacta (SNc). The majority of these neurons form striatal connection- and allows them to participate in the control of movement. The dramatic symptoms of PD as a consequence of loss of function of adult SNc neurons demonstrate this principal role. The SNc is part of the mesodiencephalic dopaminergic (mdDA) system, which besides the SNc, includes the ventral tegmental area (VTA) and the retrorubral (RR) field (Smits et al. 2006). Dopamine neurons of the VTA with their efferents to the nucleus accumbens, the dorsal striatum, the cortex, and other limbic brain areas are involved in the control of emotional behaviors and reward. Therefore, the mesolimbic dopamine system has been implicated in addictive and affective disorders such as schizophrenia and depression. The main problem neurologists are exposed to is the apparent selective degeneration of the SNc dopamine neurons in PD. Apparently, these neurons are built and maintained in such a way that they possess a selective vulnerability. The difference between dopamine neurons of the SNc and VTA is suggested to root in differences in molecular makeup, originating from different differentiation routes during embryonic development (Smidt and Burbach 2007; Smits et al. 2006). Although subset-specific differentiating processes have remained undescribed, a clear initial observation in the Pitx3 knockout has hinted at a subset-specific role for Pitx3 (Pitx family: Pitx1 (Szeto et al. 1999), Pitx2 (Campioni et al. 1999; Alward et al. 1998; Smidt et al. 2000a) and Pitx3 (Smidt et al. 1997; Semina et al. 1997) in the development and maintenance of SNc neurons (Hwang et al. 2003; Smidt et al. 2004a; Nunes et al. 2003; Burbach et al. 2003). In this chapter, the molecular background of the mdDA system

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and highlights of those molecular details that influence subset-specific vulnerability are addressed.

Vulnerability of Mesodiencephalic Dopaminergic (mdDA) Neurons

The hallmark of PD is the loss of signaling in the nigrostriatal pathway as a consequence of selective loss of SNc neurons. The discovery of genetic mutations leading to dominant and juvenile forms of PD has indicated that protein turnover and misfolding errors add substantially to the initiation of neuronal cell loss, in addition to earlier described oxidative stress and neurotoxin exposure (Mizuno et al. 2008). Other lines of investigation and the analysis of mouse models have initiated the discovery of selective vulnerability of SNc as observed in PD. Dosage differences of the homeodomain transcription factor *Engrailed* (*En1/2*) in specific mutant mice display a remarkable similarity in SNc cells loss (Simon et al. 2001, 2004, 2005; Sonnier et al. 2007) as observed in human Parkinsonian patients. An additional link between *Engrailed* and PD is the regulation of the α -synuclein gene. It has been described that *Engrailed* regulates the expression of this protein (Simon et al. 2001). Changes in *Engrailed* dose might influence the regulation of α -synuclein and thereby the potency to form aggregates in SNc neurons. However, the loss of *Engrailed* would lower the α -synuclein presence, which would lower the change of aggregate formation. Until the function of α -synuclein is described in SNc neurons, this issue will remain to be solved. Interestingly, a function in membrane trafficking has been suggested, which would indicate that loss of function due to lower gene dosage of *Engrailed* would hamper proper trafficking of proteins toward the membrane (Sidhu et al. 2004; Dauer and Przedborski 2003). The suppressed trafficking of the dopamine transporter (DAT) has been suggested to increase the intercellular reuptake of DA itself, leading to more toxic metabolites in the SNc neurons Sidhu et al. 2004; Dauer and Przedborski 2003). This pathological mechanism is tempting, since a high level of DAT is present only in the SNc, possibly explaining the higher vulnerability of the SNc to a lower *Engrailed* dose.

Another mouse mutant, the weaver (wv) mouse (Schmidt et al. 1982; Hess 1996), shows selective loss of SNc neurons and cerebellar granule cells. The mutant phenotype is caused by a gain of function mutation in the *Girk2* gene causing a constitutive and nonselective Na^+ influx. The dominant expression of *Girk2* in SNc neurons compared with VTA neurons will enhance the vulnerability of SNc neurons to this mutation, although other metabolic differences are likely to have an additional deteriorating effect. A clear effect of the wv

mutation is the altered Ca^{2+} homeostasis as a result of lower K^+ levels. It has been described in cerebellar granule cells that the consequential lower free Ca^{2+} in the cell can lead to cell death (Levick et al. 1995). Similar mechanisms may act in SNc neurons, especially since Ca^{2+} is used to generate the pacemaker activity of these neurons (Surmeier 2007). Therefore, Ca^{2+} homeostasis is even more critical in SNc neurons in addition to the vulnerability that is caused by the pacemaker activity itself in terms of metabolic strain due to the continuous activity of ATP-driven membrane Ca^{2+} transporters (Surmeier 2007). An additional problem is caused by the physiological properties and the presence of K-ATP channels. The subunit composition and the presence of these channels in the SNc have been shown to increase the vulnerability toward neurotoxins and lead to K-ATP channel activation as a consequence of mitochondrial uncoupling resulting in electrophysiological inactivation (Liss et al. 1999, 2005).

Recently, the differential expression of G-substrate (Chung et al. 2005a) (highest in VTA (A10) neurons; an endogenous inhibitor of Ser/Thr protein phosphatases) was shown to be correlated with neuronal protection against toxic insults (Chung et al. 2007). This suggests that the relative absence of this protein makes the SNc more vulnerable toward toxins compared with VTA neurons and provides a new lead to enhance the protection of SNc neurons.

Neurotrophic Support of mdDA Neurons

The selective vulnerability of the SNc as highlighted by the dramatic neuronal loss in PD has been the driving force behind the research efforts to understand maintenance and trophic support for mdDA neurons. Many trophic factors have been identified (Numan et al. 2005) and in Table 1 the proteins are listed that have been reported to have a neurotrophic effect on mdDA neurons. Most of the data on functional neurotrophic support are based on in vitro data and in addition, no specific SNc neuronal support has been described. A clear suggestion for the in vivo role of glial cell line-derived neurotrophic factor (GDNF) signaling has been the analysis of the conditional GDNF receptor (RET) knockout (Kramer et al. 2007). In this study, it was shown that RET signaling in DAT expressing neurons is essential for the long-term maintenance of mdDA neurons. Owing to the nature of the cyclization recombinase (CRE) induction (CRE was driven by the *DAT* locus), the apparent specific SNc support may have been biased, since it is known that *DAT* expression is high in the SNc and fairly low in the VTA. Experiments using a more balanced *CRE* induction system (for example *Pitx3-CRE*) might overcome this issue.

The role of BDNF and NT3, 4, and 5 was clearly established in in vivo models either by ablation of the neurotrophin

Table 1 Neurotrophic factors for mdDA neurons and their receptors

Neurotrophic factor	Receptor
Brain derived Neurotrophic factor (Bdnf) Hyman et al. (1991), Beck (1994)	TrkB & P75 receptor Zachary C Baquet et al. (2005), von Bohlen und Halbach et al. (2005)
Glial cell line derived neurotrophic factor (Gdnf) Sariola and Saarma (2003), Tomac et al. (1995)	(a) Gdnf family receptor alpha (GfR-alpha(1))/RET receptor tyrosine kinase (cRET) Sariola and Saarma (2003) (b) GfR-alpha/NCAM Sariola and Saarma (2003)
Neurturin (NRTN) Sariola and Saarma (2003), Krieglstein (2004), Horger et al. (1998)	GfR-alpha (2)/cRET Sariola and Saarma (2003)
Neublastin/Artemin (ARTN) Sariola and Saarma (2003), Krieglstein (2004)	GfR-alpha (3)/cRET Sariola and Saarma (2003)
Persephin (PSPN) (Sariola and Saarma 2003); Krieglstein (2004)	GfR-alpha(4)/cRET (Sariola and Saarma 2003)
neurotrophin-3 (NT-3) Espejo et al. (2000), Hagg (1998), Hyman et al. (1994)	TrkC von Bohlen und Halbach et al. (2005)
Growth/differentiation factor 5 (Gdf-5) Wood et al. (2005), O'Keeffe et al. (2004)	BMP receptor type 1 (BMPR-Ib) Wood et al. (2005), O'Keeffe et al. (2004)
neurotrophin-4/5 (NT-4/5) Meyer et al. (2001), Lingor et al. (2000), Altar et al. (1994), Hyman et al. (1994)	TrkB von Bohlen und Halbach et al. (2005)
Transforming growth factor-beta (Tgf-beta) Poulsen et al. (1994), Krieglstein et al. (1995a, b, 1996), Farkas et al. (2003), Roussa et al. (2004)	Tgf-beta receptor
Transforming growth factor alpha (Tgf-alpha) Alexi and Hefti (1993)	Egf/Tgf-alpha receptor Chalazonitis et al. (1992)
Neuregulin-1 (Nrg-1) Yurek et al. (2004), Segni et al. (2005)	ErbB3 & 4 receptor Thuret et al. (2004), Segni et al. (2005)
Bone morphogenetic proteins (BMPs) Jordan et al. (1997), Brederlau et al. (2002), Kerstin Krieglstein (2004)	BMP receptor type I & II Nohe et al. (2004)
Heparin-binding epidermal growth factor (HB-EGF) Iwakura et al. (2005), Hanke et al. (2004), Farkas and Krieglstein (2002)	ErbB1 Lin and Freeman(2003)
Fibroblast growth factor-2 (Fgf-2) Timmer et al. (2004), Reuss and Unsicker (2000), Shults et al. (2000), Caldwell and Svendsen (1998)	FgfR1 Stachowiak et al. (1997)
Mesencephalic astrocyte-derived neurotrophic factor (MANF) Peterson and Nutt (2008), Petrova et al. (2003), Zhou et al. (2006)	unknown
conserved dopamine neurotrophic factor (CDNF) Peterson and Nutt (2008), Lindholm et al. (2007)	unknown

itself or by ablation of their cognate receptors TrkB and TrkC (Baquet et al. 2005; von Bohlen und Halbach et al. 2005). In both the models, the survival of mdDA neurons is clearly affected. However, the relative contribution to the cell loss of the SNc in comparison with the VTA is not described. This leaves the discussion open whether TrkB and C signaling influence SNc maintenance specifically.

The protective role of transforming growth factor β (Tgf- β) was described extensively in vitro (Krieglstein et al. 1995b; Roussa et al. 2004; Krieglstein et al. 2004; Farkas et al. 2003; Krieglstein et al. 1995a and in vivo (Roussa et al. 2006). The in vivo data hint at a function in mdDA induction and differentiation. However, the described cross-talk between Tgf- β and GDNF signaling still hints at a genuine maintenance role.

Taken together, there are many neurotrophic factors described that influence the survival of mdDA neurons. A clear lack in the analysis is the specificity of these factors toward SNc neurons. Additional genetic models in which SNc and VTA are carefully compared will provide more evidence to solve this.

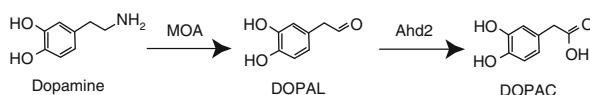
Molecular Coding of Mesodiencephalic Neurons

Specific vulnerability of subsets of mdDA neurons may rely on differences in molecular signature. This suggests that

specific differences exist in differentiation programs of mdDA subsets, generated by different developmental programs (Smits et al. 2006). Among the earliest events fundamental to mdDA neuronal development is the specification of the permissive region to allow dopamine neurons to be generated. Crucial in formation of the mdDA region is the positioning of the mid-hindbrain border, the isthmus. The signaling emerging from the isthmus (Fgf8) together with signaling from the notochord (Shh) designate, at a specific signaling intersection point, the region where mdDA neurons are born (Hynes and Rosenthal 1999; Hynes et al. 1995, 2000). Changing the position of the isthmus, through manipulation of key transcription factors such as Otx2 and Gbx2 (Simeone 2002; Rhinn and Brand 2001; Simeone 2002; Glavic et al. 2002), indirectly influences the emergence of mdDA neurons through the ablation (Acampora et al. 1999, 2000, 2001; Simeone 2002; Acampora et al. 2005) or expansion (Joyner et al. 2000; Wassarman et al. 1997; Millet et al. 1999) of the midbrain being the main site of mdDA neuronal generation. After this initial regional specification, a mix of transcription factors has been implicated, through mouse models, in the development of mdDA neurons (Table 2). Long-term analysis of engrailed1/2 mutants or hypomorphs has shown that gene dosage is incremental to the survival of mdDA neuron (Sonnier et al. 2007).

Table 2 Transcription factors involved in the development of mdDA neurons

Transcription factor	Activated genes related to mdDA neurons
Pitx3 Smidt et al. (2004), Nunes et al. (2003), Hwang et al. (2003)	Ahd2, Th Jacobs et al. (2007); Maxwell et al. (2005)
Engrailed1 and 2 Simon et al. (2001), Sonnier et al. (2007), Wurst et al. (1994)	alpha-synuclein Simon et al. (2001)
Nurr1 Zetterström et al. (1997), Saucedo-Cardenas et al. (1998)	TH, VMAT2, DAT, cRET Wallén et al. (2001), Smits et al. (2003)
Lmx1b Smidt et al. (2000)	unknown
Lmx1a Andersson et al. (2006a,b)	Msx1, Ngn2 Andersson et al. (2006a, b)
Ngn2 Andersson et al. (2006a, b) Kele et al. (2006)	Sox2 Kele et al. (2006)
Foxa1 and a2 Ferri et al. (2007)	Ngn2 Ferri et al. (2007)
Otx2 Puellas et al. (2003), Prakash et al. (2006), Vernay et al. (2005), Puellas et al. (2004)	unknown

**Fig. 1** Dopamine metabolism generating toxic products and detoxification by Ahd2. DOPAL 3,4-dihydroxyphenylacetaldehyde, DOPAC 3,4-dihydroxyphenylacetic acid, MOA Mono amine oxidase, Ahd2 Aldehyde dehydrogenase 2 (mouse).

The discovery of *Pitx3* was interesting, since its expression is limited to mdDA neurons (Smidt et al. 1997). The analysis of the *Pitx3* mutant showed that mainly SNc cells are dependent on *Pitx3* function, although a milder phenotype was described within the VTA (Smidt et al. 2004a,b). At that moment, the specific vulnerability was not understood and was thought to rely on other molecular pathways interacting with *Pitx3*. Recent data have shown that the subset-specific activation of the *Ahd2* gene by *Pitx3* is (partly) responsible for the subset-specific vulnerability (see the following section) (Jacobs et al. 2007). In conclusion, many transcription factors have been identified as having a role in the development of mdDA neurons (Jacobs et al. 2006). From these factors, *Pitx3* is the most appealing, generating specific mdDA cell loss in the mutant overlapping with the vulnerable SNc neuronal population.

The role of RA and RA-Generating Enzymes in Development and Maintenance of DA Neurons

The tempting observation that *Pitx3* loss leads to SNc loss has triggered the analysis of targets of *Pitx3* in relation to SNc development. From these analyses, it became clear that during development neurons are present until late stages in the *Pitx3* knockout, but the expression of *Ahd2* (McCaffery and Dräger 1994a) is lost (Jacobs et al. 2007). *Ahd2* is an aldehyde dehydrogenase that is able to convert aldehydes into acids. In the central nervous system, aldehyde dehydrogenases

are present in specific regions during development and in the adult.

Detoxification of Aldehydes

Dopamine is metabolized by monoamine oxidase (primarily MAO-A) and deaminated to 3,4-dihydroxyphenylacetaldehyde (DOPAL). The latter compound is considered to be a neurotoxin and accumulation of this metabolite has been considered as a cause of neurodegeneration as seen in PD (Marchitti et al. 2007). The reactive properties of aldehydes such as DOPAL can be reduced through the oxidation to the corresponding carboxylic acid (DOPAC, Fig. 1). This reaction is catalyzed by aldehyde dehydrogenases such as *Ahd2*. It has been suggested that reactions of DOPAL toward α -synuclein cause aggregates found in PD patients (Galvin 2006), suggesting that the vulnerability of SNc neurons is strictly correlated with the metabolic rate of dopamine turnover. In addition, it has been described, from microarray experiments that genes involved in (energy) metabolism are highly expressed in the SNc compared with the VTA (Greene et al. 2005). Moreover, microarray experiments performed on PD material suggested that an aldehyde dehydrogenase, *ALDH1A1*, is downregulated (Grünblatt et al. 2004; Mandel et al. 2005). Taken together, the presence of the dopamine metabolite DOPAL in the SNc together with the high metabolic rate and the presence of high levels of *Ahd2* may represent a critical process in keeping SNc neurons healthy. A small unbalance in the metabolism of the neurons may quickly lead to toxic episodes and consequential neuronal loss.

Retinoic Acid and SNc Development

Retinoic acid (RA), the active derivative of Vitamin A (retinol), is generated by three different dehydrogenases, *Raldh1-3* (Smith et al. 2001), after initial retinol oxidation by alcohol dehydrogenases (Westerlund et al. 2005). The presence of these enzymes is an indication of production of functional RA, as was shown in RA-reporter mice (Smith et al. 2001; Westerlund et al. 2005; McCaffery and Dräger 1994b). The expression of *Raldh1* (*Ahd2*) in the midbrain ventricular zone (Westerlund et al. 2005; Wallén et al. 1999) in early developing and adult mdDA neurons (Smith et al. 2001; McCaffery and Dräger 1994a; Galter et al. 2003) suggests that RA is locally synthesized during all the steps in development and may have a function in mdDA generation and differentiation (Chung et al. 2005b).

Besides the detoxifying role of *Ahd2* in SNc neurons, an essential role in SNc development has been described, suggesting essential molecular signature differences between SNc and VTA for their dependence on RA signaling. The subset-specific loss of SNc neurons in *Pitx3* knockout animals suggested a specific *Pitx3* dependence, in terms of survival, compared with VTA neurons. Recently, it has been described that *Pitx3* acts as an upstream activator of the *Ahd2* gene (Jacobs et al. 2007). This suggests that RA signaling, through activation of the *Ahd2* gene by *Pitx3*, is essential for the development and maintenance of SNc neurons (Fig. 2).

Importantly, the ectopic application of RA to developing *Pitx3* knockout animals largely rescues the SNc neurons.

These data imply that most neurons in the SNc depend on RA signaling during development. This dependence suggests that the described loss of *Ahd2* expression in PD patients may contribute to the loss of essential signaling mechanisms for SNc neuronal maintenance. Moreover, it has been reported that Disulfiram (an *Ahd2* inhibitor) abuse can lead to Parkinsonian features in patients (Krauss et al. 1991) and SH-SY5Y cell toxicity (Legros et al. 2004). However, it remains unclear at this moment whether RA signaling itself remains crucial in the molecular mechanism of SNc maintenance. Taken together, the subset-specific activation of the *Ahd2* gene by *Pitx3* provides a clear evidence that molecular coding differences can lead to specific vulnerability within the SNc.

mdDA Subset Signature

Microarray studies unraveling the transcriptional profile differences between SNc (A9) and VTA (a10) neuronal groups have shown that many genes are differentially expressed between these two neuronal populations (Chung et al. 2005a; Greene et al. 2005). For many of these genes, the functional relevance of the differential expression is unknown, as is whether the expression within SNc and VTA is subset specific. Based on in situ hybridization and physiological studies, the genes listed in Table 3 have been shown to exhibit subset-specific expression, not clearly mapping to SNc or VTA alone. The expression patterns of *Girk2*, *DAT*, *Ahd2*, *Sur1b*, and *G-substrate* have clearly shown a direct relationship with

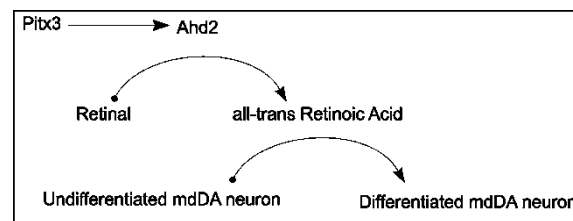


Fig. 2 Schematic representation of the regulated RA production, through initial activity of *Pitx3* and induction of *Ahd2*. The local RA production establishes the development of a subset of SNc neurons.

Table 3 Subset specific markers within the mdDA neuronal population

Gene:	Specific expression pattern:
Calbindin (Murase and McKay 2006; Alfahel-Kakunda and Silverman 1997; Haber et al. 1995; Verney et al. 2001)	Subset of VTA
<i>Girk2</i> (Chung et al. 2005; Schein et al. 1998)	Subset of SNc
<i>MGLuR1</i> (Smits et al. 2005; Kaneda et al. 2003)	Subset of SNc
<i>DAT</i> (Haber et al. 1995; Smidt et al. 2004, 2005)	Subset of SNc and VTA
<i>Ahd2</i> (Jacobs et al. 2007)	Subset of SNc and VTA
Neurotensin (Smits et al. 2004)	Small subset of SNc and VTA
<i>Sur1b</i> (Liss et al. 2005; Liss et al. 1999)	Neurons lost by the <i>wv</i> mutation
<i>G-substrate</i> (Chung et al. 2007; Chung et al. 2005)	VTA neurons

specific vulnerability and therefore indicate that molecular subset specification can determine the vulnerability of (subsets of) SNc neurons. For all the other genes listed from the initial microarray experiments, the functional significance has to be determined as well as the genetic coding of these subsets, driving the specific differentiation patterns leading to subset specification. The subset-specific activation of *Ahd2* and *Th* by the transcription factor *Pitx3* (Jacobs et al. 2007; Maxwell et al. 2005) shows that other interacting pathways exist that drive subset-specific dependence on broadly mDA neuron expressed transcription factors.

Conclusions

The data that explain the specific vulnerability of SNc neurons are scarce and incomplete. From the data summarized earlier, it is clear that mechanisms involved in metabolic activity, oxidative stress, detoxification, and molecular signature can all add to SNc vulnerability. Interestingly, more data are emerging that describe the subset specification within the mDA neuronal population (Smits et al. 2006) that supersedes the anatomical distinction between SNc and VTA. Current efforts are focusing on defining the molecular signature of mDA neuronal populations and are trying to understand how these are generated during development. Ultimately, knowledge of the different molecular mDA signatures will help us to understand why specific neurons are vulnerable to genetic or physiological burden. Moreover, at that point, new therapies can be developed to stop disease progression in the case of Parkinson's disease.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 4

The Nigrostriatal Pathway: Axonal Collateralization and Compartmental Specificity

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Abstract This paper reviews two of the major features of the nigrostriatal pathway, its axonal collateralization, and compartmental specificity, as revealed by single-axon labeling experiments in rodents and immunocytochemical analysis of human postmortem tissue. The dorsal and ventral tiers of the substantia nigra pars compacta harbor various types of neurons the axons of which branch not only within the striatum but also in other major components of the basal ganglia. Furthermore, some nigrostriatal axons send collaterals both to thalamus and to brainstem pedunculopontine tegmental nucleus. In humans, the compartmental specificity of the nigrostriatal pathway is revealed by the fact that the matrix compartment is densely innervated by dopaminergic fibers, whereas the striosomes display different densities of dopaminergic terminals depending on their location within the striatum. The nigral neurons most severely affected in Parkinson's disease are the ventral tier cells that project to the matrix and form deep clusters in the substantia nigra pars reticulata.

Keywords Basal ganglia • Parkinson's disease • Single-cell labeling • Striatal compartments • Substantia nigra • Tyrosine hydroxylase

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Abbreviations

A	Anterior
ac	Anterior commissure
CB	Calbindin
CC	Corpus callosum
CN	Caudate nucleus
cp	Cerebral peduncle
CPu	Caudate-putamen
D	Dorsal
DA	Dopaminergic
ENK	Enkephalin
EP	Entopeduncular nucleus
FF	Fields of Forel
Fr 2	Frontal cortex, area 2
FStr	Fundus striate
GP	Globus pallidus
ic	Internal capsule
ir	Immunoreactive
L	Lateral
LAMP	Limbic system-associated membrane protein
LPB	Lateral parabrachial nucleus
LV	Lateral ventricle
M	Striatal matrix compartment
ml	Medial lemniscus
MPB	Medial parabrachial nucleus
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MT	Medial terminal nucleus of the accessory optic tract
NADPH-d	Nicotinamide adenine dinucleotide phosphate reduced-diaphorase
PC	Paracentral thalamic nucleus
PD	Parkinson's disease
PnO	Pontine reticular nucleus
Put	Putamen
RN	Red nucleus
RRF	Retrorubral field
S	Striosome

SS	Subcallosal streak
scp	Superior cerebellar peduncle
SNc	Substantia nigra <i>pars compacta</i>
SNr	Substantia nigra <i>pars reticulata</i>
SS	Subcallosal streak
STN	Subthalamic nucleus
SubI	Subincertal nucleus
Th	Thalamus
TH	Tyrosine hydroxylase
VL	Ventrolateral thalamic nucleus
VM	Ventromedial thalamic nucleus
VTA	Ventral tegmental area
ZI	Zona incerta

Introduction

The basal ganglia work in concert with the cortex to orchestrate and execute planned, motivated behaviors requiring motor, cognitive, and limbic circuits. The striatum is the main input structure of the mammalian basal ganglia receiving projections mainly from the cortex and the thalamus (Haber and Johnson Gdowski 2004). The striatum is also abundantly innervated by fibers from the substantia nigra. The nigrostriatal dopaminergic pathway is crucial in the organization of the basal ganglia realm, modulating a broad range of behaviors from learning and working memory to motor control (Haber and Johnson Gdowski 2004).

The substantia nigra is a flattened oval structure on the dorsal aspect of the cerebral peduncle. On the basis of cytoarchitectonic and chemical criteria, the substantia nigra is subdivided into two cell groups: the pars compacta (SNc) and the pars reticulata (SNr) (Olszewski and Baxter 1954; Poirier et al. 1983; Francois et al. 1985; Halliday and Törk 1986). This subdivision into two components is supported by the chemical anatomy of this nuclear complex. Thus, the SNc is mainly constituted by dopaminergic cell bodies that project massively to the striatum, while the SNr contain GABAergic neurons the axons of which innervate the thalamus, the superior colliculus, and the pedunculopontine tegmental nucleus (Cebrián et al. 2005). Another additional subdivision of the substantia nigra named pars lateralis has also been reported, but the neurons of this region have been shown to resemble those of the SNr in their somatodendritic morphological features and axonal branching patterns (Cebrián et al. 2005).

The aim of this paper is to provide an overview of the organization of the SNc and its efferent projections. The data presented here stem from both experimental studies in rats and cytochemical analyses in human postmortem tissue. The first part of the paper deals with the various subdivisions of the dopaminergic (DA) neurons of the SNc in rodents and primates and subsequently with the patterns of axonal

branching of neurons located in various sectors of the rodent SNc. The second part of the paper focuses first on the compartmental organization of the striatum and then on the relationship between the striatum and the nigrostriatal pathway, as revealed by single axon reconstructions in the rat and immunohistochemical staining applied to human post-mortem tissue.

The Substantia Nigra Pars Compacta in Rats and Primates

According to their chemical properties, the DA neurons of the SNc in rodents and primates are subdivided in two main populations of cells named dorsal and ventral tiers (Gerfen et al. 1987a, b; Lynd-Balta and Haber 1994). The DA neurons of the dorsal tier differ from those of the ventral tier by the fact that they contain the calcium-binding protein calbindin (CB) (Gerfen et al. 1987b). The most widely used organizational scheme of the nigrostriatal system holds that neurons in the dorsal and ventral tiers of the SNc project to different striatal compartments and this aspect will be dealt with in another section of this review.

The DA neurons of the dorsal tier are located preferentially in the rostral and dorsal sector of the SNc and they form a continuous band with the ventral tegmental area (VTA) and the retrorubral field (RRF) (Fig. 1) (Gerfen et al. 1987a; Haber and Fudge 1997). In contrast, the ventral tier neurons are widely scattered within the SNc. Some of these CB-negative DA neurons are located just above the dorsal edge of the SNr (see ventral tier SNc outside SNr in Fig. 1). More caudally, the ventral tier neurons are scattered within the SNr (see ventral tier SNc within SNr in Fig. 1) and some of them form typical cell clusters deeply embedded in the SNr (see cell clusters of ventral tier SNc in Fig. 1) (Prensa and Parent 2001). The ventral tier neurons that form clusters in the deep portion of the SNr correspond to the cell columns (or fingers) described in primates (see below) and are considered as displaced SNc neurons (Van der Kooy 1979; Guyenet and Crane 1981; Lynd-Balta and Haber 1994; Fallon and Loughlin 1995; Joel and Weiner 2000).

In primates, DA neurons can be distinguished from the underlying SNr by their content of neuromelanin, a dark pigment that accumulates in catecholamine neurons, especially those of the SNc in human brain (Braak and Braak 1986). The SNc in primates is also subdivided into a CB-positive dorsal tier (the α group or pars dorsalis) contiguous to the VTA (Fig. 2a–c) and a CB-negative ventral tier that includes a densocellular region (the β group) and cell columns (the γ group) (Fig. 2d, e) (Poirier et al. 1983; Francois et al. 1985; Halliday and Törk 1986; Haber et al. 1995;

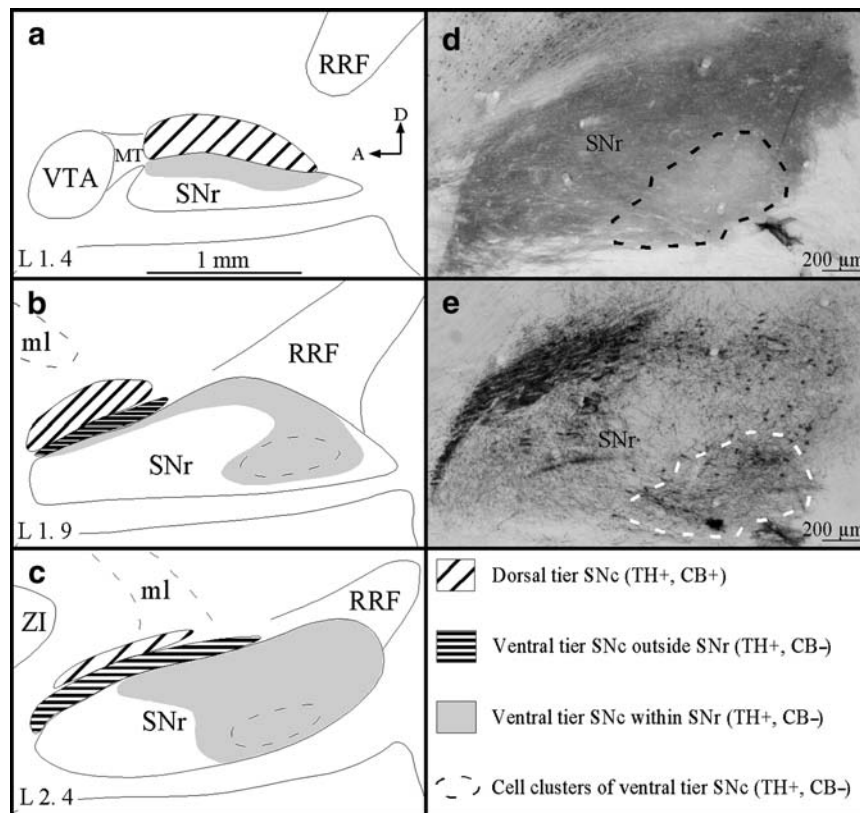


Fig. 1 (a–c) Drawings showing the localization and extent of the dorsal and ventral tiers of the SNc on three sagittal sections of the rat midbrain. The drawings are set out in a mediolateral order, and the laterality (L) of each section, according to the atlas of Paxinos and Watson (1986), is indicated in the bottom left. The exact location of the dorsal tier and the various subdivisions of the ventral tier of the SNc are identified by various hatched and gray areas, the significance of which is explained in the bottom right. (d, e) Low-power photomicrographs of two adjacent parasagittal sections of the substantia nigra stained for calbindin (CB) (d) and tyrosine hydroxylase (TH) (e) respectively. They show one of the typical SNr oval sector (dashed line) characterized by a CB-poor neuropil and clustered TH-positive neurons. The mediolateral level corresponds approximately to that of the drawing in (b). For abbreviations see abbreviations list. Figure slightly modified from Prensa and Parent (2001)

Giménez-Amaya et al. 2004). The ventral tier cells are more vulnerable to degeneration in Parkinson's disease (PD) and to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicity, while the CB-positive dorsal tier cells are selectively spared (Fallon and Moore 1978; Gerfen et al. 1985; Lavoie and Parent 1991; Pifl et al. 1991; Parent and Lavoie 1993; Haber et al. 1995).

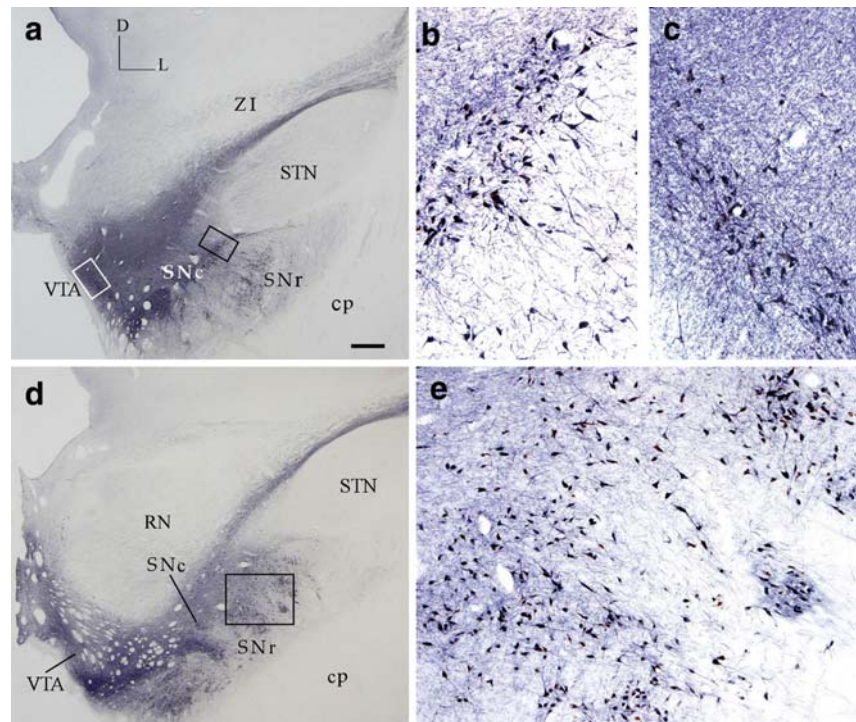
The Nigrostriatal System: A Highly Collateralized Mesencephalic Dopaminergic Pathway

The nigrostriatal pathway courses from the SNc to the dorsal striatum through the medial forebrain bundle. This projection system exerts upon the striatum a profound influence that affects both motor and motivational aspects of behavior (Gerfen and Wilson 1996). The nigrostriatal pathway is part of a larger mesotelencephalic system that originates from

DA neurons scattered in the RRF, SNc, and VTA, corresponding respectively to groups A8, A9, and A10 of Dahlström and Fuxe (1964), see also Björklund and Lindvall (1984). Neurons of A9 and A8 groups contribute to the nigrostriatal system, whereas those of the A10 supply the mesolimbocortical system, which innervates structures such as the amygdala, septum, olfactory tubercle, and the prefrontal/anterior cingulate cortices (Berger et al. 1974; Lindvall et al. 1977; Fallon and Moore 1978; Fallon and Loughlin 1985, 1987; Gerfen et al. 1987b; Hontanilla et al. 1996).

Our knowledge of the anatomical and functional organization of the nigrostriatal pathway has greatly improved in recent years thanks to a series of studies of the axonal collateralization of nigrostriatal neurons located in different sectors of the SNc. The use of neuronographic methods based on the axonal transport of various molecular markers has revealed an abundance of SNc neurons endowed with a highly patterned set of axon collaterals, and this feature is shared by virtually all of the major components of the basal ganglia in rodents and primates (Gauthier et al. 1999; Parent

Fig. 2 (a, d) Low-power photomicrographs of tyrosine hydroxylase (TH)-immunostaining in the human midbrain, as viewed in the coronal plane. (b, c) High-power view of the TH-positive fibers and cells of the dorsal tier of the substantia nigra pars compacta (b) and ventral tegmental area (c). (e) High-power view of the densocellular region and the cell columns of the ventral tier of SNc. Scale bar: a and d, 1 mm; b, c and e, 300 μ m. For abbreviations see abbreviations list



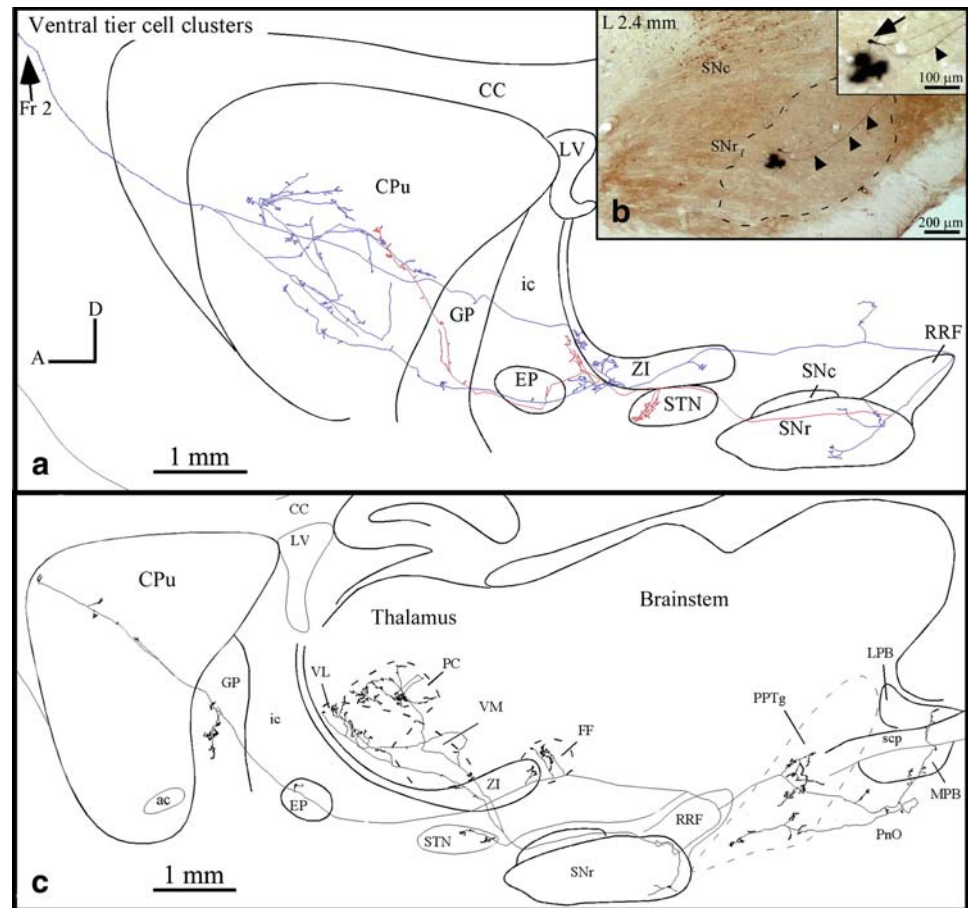
et al. 2000; Prensa and Parent 2001; Parent and Parent 2004, 2006; Cebrián et al. 2005). In humans, the nigrostriatal axons visualized with tyrosine hydroxylase (TH)-immunohistochemistry were also seen to provide collaterals to the globus pallidus and the subthalamic nucleus (Cossette et al. 1999; Hedreen 1999; Prensa et al. 2000).

The analysis of the efferent projections of single neurons located in either the dorsal or the ventral tiers of the SNc in the rat has revealed complex patterns of axonal arborization (Prensa and Parent 2001). This multifaceted system targets, in addition to the striatum, several extrastriatal basal ganglia structures, such as the subthalamic nucleus and the globus pallidus, as well as the intralaminar and ventral thalamic nuclei and other basal forebrain structures (Fig. 3). Although the ability of targeting extrastriatal structures is shared by dorsal and ventral tier neurons, the degree of axonal branching at the extrastriatal level is higher in ventral tier neurons. Furthermore, the highest degree of axonal branching outside the striatum is provided by neurons forming the cell clusters of the ventral tier (Fig. 3). The fact that many of these clustered ventral tier neurons send few poorly branched collaterals in the striatum suggests that the degree of axonal branching at striatal level might be inversely proportional to the degree of axonal branching at extrastriatal level. Both dorsal and ventral tier neurons emit intranigral axon collaterals that arborize profusely not only within the SNc but also within the SNr, which is one of the major output structures of the basal ganglia. These local axon collaterals are abundant

in the dorsal aspect of the SNr, a region that harbors projection neurons with especially large dendritic fields and widely branched axons (Cebrián et al. 2007). Although the intranigral axon collaterals arise from a minority of SNc neurons, the intranigral collateral network has been shown to be well developed in a variety of mammalian species, including human and nonhuman primates (Preston et al. 1981; Yelnik et al. 1987).

Neurons that are most prone to degeneration in PD are the ventral tier neurons lying in the most ventral and lateral aspects of the substantia nigra (Gibb et al. 1990; Fearnley and Lees 1991; Gibb and Lees, 1991; Rinne 1993). These neurons contain neuromelanin pigments and degenerate massively in sporadic PD (Hirsch et al. 1988). The axons of these cells arborize not only in the striatum but also in extrastriatal structures such as the subthalamic nucleus, various thalamic nuclei, or the pedunculopontine tegmental nucleus (Fig. 3) (Prensa and Parent 2001). Collateral projections appear to target functionally homologous striatal and thalamic subdivisions (Kolmac and Mitrofanis 1998), providing the potential for subpopulations of SNc neurons to modulate behaviors independently within restricted domains. Observations in PD monkeys have revealed that nigrostriatal DA neurons arborizing profusely in the striatum are much more vulnerable to degeneration than nigral neurons with an axon that branches abundantly in nonstriatal structures and relatively little in the striatum (Parent and Parent 2006).

Fig. 3 (a, c) Camera lucida drawings of two ventral tier SNc axons, the neurons of which lie in a cell cluster located in the SNr of the rat, as viewed in the sagittal plane. (b) Photomicrograph of the injection site in the ventral portion of the SNr surrounded by the neuron, the axon of which is illustrated in (a), and the arrowheads point to the initial segment of the axon. The inset in (b) offers a high-power view of the neuron (arrow) and part of its axon (arrowhead). Besides the striatum, both nigrostriatal neurons innervate a great variety of structures such as the globus pallidus, the subthalamic nucleus, ventral and intralaminar thalamic nuclei, and the pedunculopontine tegmental nucleus. For abbreviations see abbreviations list. Figure modified from Prensa and Parent (2001)



Striatal and Striosomal Heterogeneities

The striatum is composed of two distinct compartments, the striosomes (or patches) and the surrounding extrastriosomal matrix, which are known to have distinct chemical compositions and input/output connections (Gerfen et al. 1987a, b; Graybiel 1990; Gerfen 1992; Holt et al. 1997; Prensa et al. 1999; Bernácer et al. 2008). Although the function of this compartmentalization is still poorly understood, this dual organization of the striatum is one of the most important features for understanding the striatal relationships with the dopaminergic system.

A histochemical compartmentalization of the striatum has been demonstrated for a variety of neuropeptides and transmitter-related enzymes. Striosomes are characterized by high levels of opiate receptors, substance P, enkephalin (ENK), neurotensin, and limbic system-associated membrane protein (LAMP), and are considered to carry neural information from limbic-related nuclei (Graybiel 1990; Eblen and Graybiel 1995; Prensa et al. 1999; Bernácer et al. 2008) (Fig. 4). The striosomal compartment includes the so-called subcallosal streak, a thin but rather extended

portion of the striatum that forms a rim subjacent to the corpus callosum (Prensa and Parent 2001; Lévesque et al. 2004). The extrastriosomal matrix displays high levels of acetylcholinesterase, calcium-binding proteins, choline acetyltransferase, nicotinamide adenine dinucleotide phosphate reduced-diaphorase (NADPH-d), and TH, and is the target of thalamic projections.

The existence of the striosome/matrix compartmentalization of the striatum has been reported in various species including rodents, cats, nonhuman primates, and humans. In addition, a further subdivision of the striosomal compartment has been described in the human brain (Prensa et al. 1999). A detailed analysis of the distribution of a wide variety of neurochemical markers revealed that the striosomes are composed of two chemically distinct regions, a core and a periphery, which are likely to play a different role in the functional organization of the human striatum (Prensa et al. 1999) (Fig. 4). The chemical heterogeneity of striosomes is best exemplified by analyzing the distribution of LAMP, a reliable marker of limbic system connections (Levitt 1984), while taking into account the fact that striosomes are reportedly the preferential targets of limbic striatal

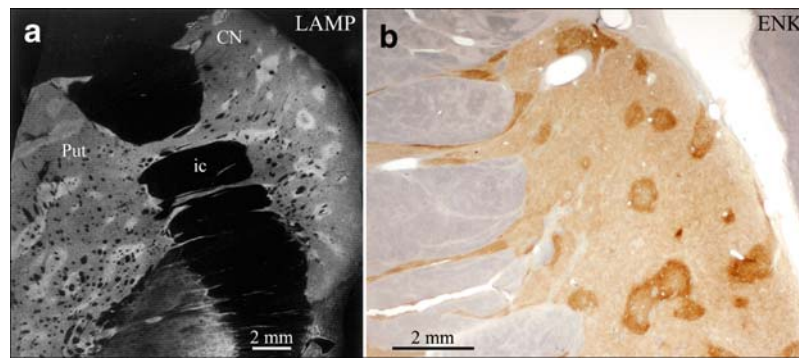


Fig. 4 (a) Direct “negative” print of a coronal section of the human caudate nucleus (CN) and the putamen (Put) stained for the visualization of limbic system-associated protein (LAMP) immunoreactivity. (b) High-power view of another coronal section of the human CN immunostained for enkephalin (ENK). The striosomes are clearly delineated by their intense immunoreactivity for LAMP and ENK. Observe that the immunostainings for these two markers within the striosomes are intense in the peripheral ringed area that surrounds a poorly stained central core

afferents (Gerfen 1984; Donoghue and Herkenham 1986; Graybiel 1990). The fact that LAMP immunostaining is much more intense at the periphery than in the core of striosomes suggests that striatal limbic afferents arborize more profusely in the peripheral zone than in the core of striosomes in humans (Fig. 4a). The peripheral region of striosomes displays a more intense ENK immunoreactivity than the central core (Fig. 4b). If the density of this immunostaining reflects the number of ENK-ir neurons intrinsic to human striatum, as it has been demonstrated in cats and monkeys treated with colchicine (Graybiel and Chesselet 1984; Martin et al. 1991), this finding would indicate that the peripheral region of striosomes harbors a greater population of ENK-immunoreactive (-ir) neurons than the core. This ENK-rich periphery may thus be an important source of enkephalinergic striatopallidal fibers, which terminate preferentially in the lateral segment of the globus pallidus.

There are certain variations in the chemical features of striosomes that occur along the anterior–posterior axis of the striatum. This is especially noticeable in TH-immunostained sections, since striosomes located in the anterior aspect of the striatum lack TH whereas those located in the posterior aspect show a core region with a very high density of TH-ir fibers. Furthermore, the core of striosomes stains more intensely for TH than the periphery, a finding suggesting that the dopaminergic innervation of certain striosomes in the human is largely oriented toward the core region (see the following section).

The Compartmental Organization of the Nigrostriatal Pathway

The most widely accepted morphological and functional scheme of the nigrostriatal system holds that dopaminergic

projections from dorsal tier neurons are directed mainly to the matrix compartment, whereas projections from the ventral tier target the striosomes (or patch compartment) (Gerfen et al. 1985, 1987a, b; Jimenez-Castellanos and Graybiel 1987; Langer and Graybiel 1989; Gerfen 1992; Haber and Fudge 1997; Song and Haber 2000). This view is, nevertheless, difficult to reconcile with the fact the striatal dopaminergic denervation in PD is massive and involves both patches and matrix. It also does not fit with the idea that the dopaminergic neurons that are most severely affected in this disease (the numerous ventral pigmented neurons) are those that are said to specifically innervate the smallest striatal compartment (the striosomes), which represents only about 20% of the total striatal volume (Johnston et al. 1990). It should be noted that the latter organization scheme of the nigrostriatal system was proposed on the basis of results obtained by means of bulk injections of anterograde and/or retrograde axonal tracers. Because of the thinness of the dorsal and ventral tiers of the SNc, studies with more refined neuroanatomical procedures, such as single-cell labeling techniques, are required to validate this scheme and to further our knowledge of the compartmental organization of the nigrostriatal pathway.

The results of a detailed single-axon labeling study of the relationship between dorsal and ventral tier nigral neurons and striosome/matrix compartments in rats supported the existence of a compartmental mode of organization of the nigrostriatal system (Prensa and Parent 2001). The latter study has revealed that most of the dorsal tier SNc neurons innervate almost exclusively the matrix, where they arborize into either one specific area or multiple discontinuously areas scattered dorsoventrally or rostrocaudally within the matrix compartment (see dorsal tier type 1 in Table 1). The study also reported a few dorsal tier SNc neurons that arborize in both matrix and striosomes, a finding that seems to be contradictory to the rule of compartmental specificity (see dorsal tier type 2 in Table 1; Fig. 5). However, the fact that

Table 1 Axonal branching patterns of nigrostriatal cells at striatal and extrastriatal levels in the rat

Nigral sector, number of labeled axons (n), and cell type	Axonal branching: sites and degree ^a				
	CPu			FStr	Extrastriatal structures
	M	S	SS		
Dorsal tier (n=19)					
Type 1 (n=12)	++/+++	-/+	-/+	-	-/+
Type 2 (n=3)	++/+++	++/+++	-/+	-	+/>++
Type 3 (n=4)	-	-	-/+	++/+++	-/+
Ventral tier (n=23)					
Outside SNr (n=12)	-/+	+	-/+	-	-/+
Within SNr (n=7)	+	++/+++	-	-	+/>++
Cell clusters (n=4)					
1	+++	-	+	-	++
2	+	-	+	-	+++
3	-	-	+	-	+++
4	-	+	-	-	+++

CPu Caudate-putamen, FStr Fundus striate, M Striatal matrix compartment, S Striosome; SS subcallosal streak

^aDegrees of axonal branching: - none, + weak, ++ moderate, +++ high. From Prensa and Parent (2001)

the innervation of the two striatal compartments by these neurons appears to derive from different axonal branches supports the idea of the existence of a compartmental selectivity for each branch of these axons (Fig. 5).

The ventral tier of the SNc has been proved to comprise neurons with many different patterns of axonal branching within the striatum. In fact, ventral tier neurons lying above the dorsal limit of the SNr innervated striosomes (see ventral tier outside SNr in Table 1), whereas neurons lying within the SNr target striosomes as well as the surrounding extra-striosomal matrix (Table 1). Interestingly, the ventral tier neurons that form clusters in the deep portion of the SNr innervate the matrix massively and the striosomes only weakly or not at all (Table 1). The fact that the most ventrally located ventral tier neurons innervate the matrix compartment should be taken into consideration, since these neurons are more vulnerable both in PD and in MPTP-treated monkeys and their degeneration could therefore be the cause of the loss of dopaminergic terminals in both matrix and striosomes.

The compartmental organization of the dopaminergic innervation in the human striatum can be observed in TH-immunostained material. As previously mentioned, the TH-ir neuropil is denser in the matrix compartment than in the striosomes (Holt et al. 1997; Prensa et al. 1999). However, this pattern of staining does not apply to the entire length of the striatum, since the dorsolateral aspect of the post-commissural putamen contains striosomes densely stained for TH (Prensa et al. 1999; Bernácer et al. 2008). Furthermore, as we have mentioned in the previous section, these posterior striosomes express a denser TH-immunostaining in their center (or core region) than in their periphery. A detailed analysis of the TH-immunoreactivity of the human striatum revealed that the core region of striosomes and the matrix harbor a dense field of isolated varicosities

reminiscent of terminal boutons as well as numerous long and thick fibers (Prensa et al. 1999). Many of the TH-positive fibers observed in the core region of striosomes traverse the boundary of striosomes and course for long distances within the matrix. In adjacent sections stained for CB, the same striosomes contain CB-positive fibers that follow the same trajectory as the TH-ir ones. Assuming that these fibers are coursing from striosomes to the matrix and indeed coexpress TH and CB, it might be hypothesized that in humans the nigrostriatal axons course through the striosomal network, including those that arise from the dorsal tier of the SNc and target the matrix compartment.

Concluding Remarks

The pathological hallmark of PD is a massive loss of the DA neurons of the SNc. A detailed knowledge of the connections of these neurons is essential for understanding the pathophysiology of Parkinsonism. Immunohistochemical studies of the DA innervation of the human basal ganglia and single-cell labeling studies of the nigrostriatal pathway in the rat have revealed that, in contrast to previous beliefs, the nigrostriatal projection system is not a monolithic entity. Instead, this important projection system is made of a great variety of axonal types, each having its own site of origin and its specific target structures. Irrespective of the location of the cell body in the SNc (dorsal or ventral tier), the nigrostriatal axons provide collaterals that innervate all the components of the basal ganglia. However, nigrostriatal axons that emit collaterals to the thalamus and to the pedunculopontine tegmental nucleus arise mostly from ventral

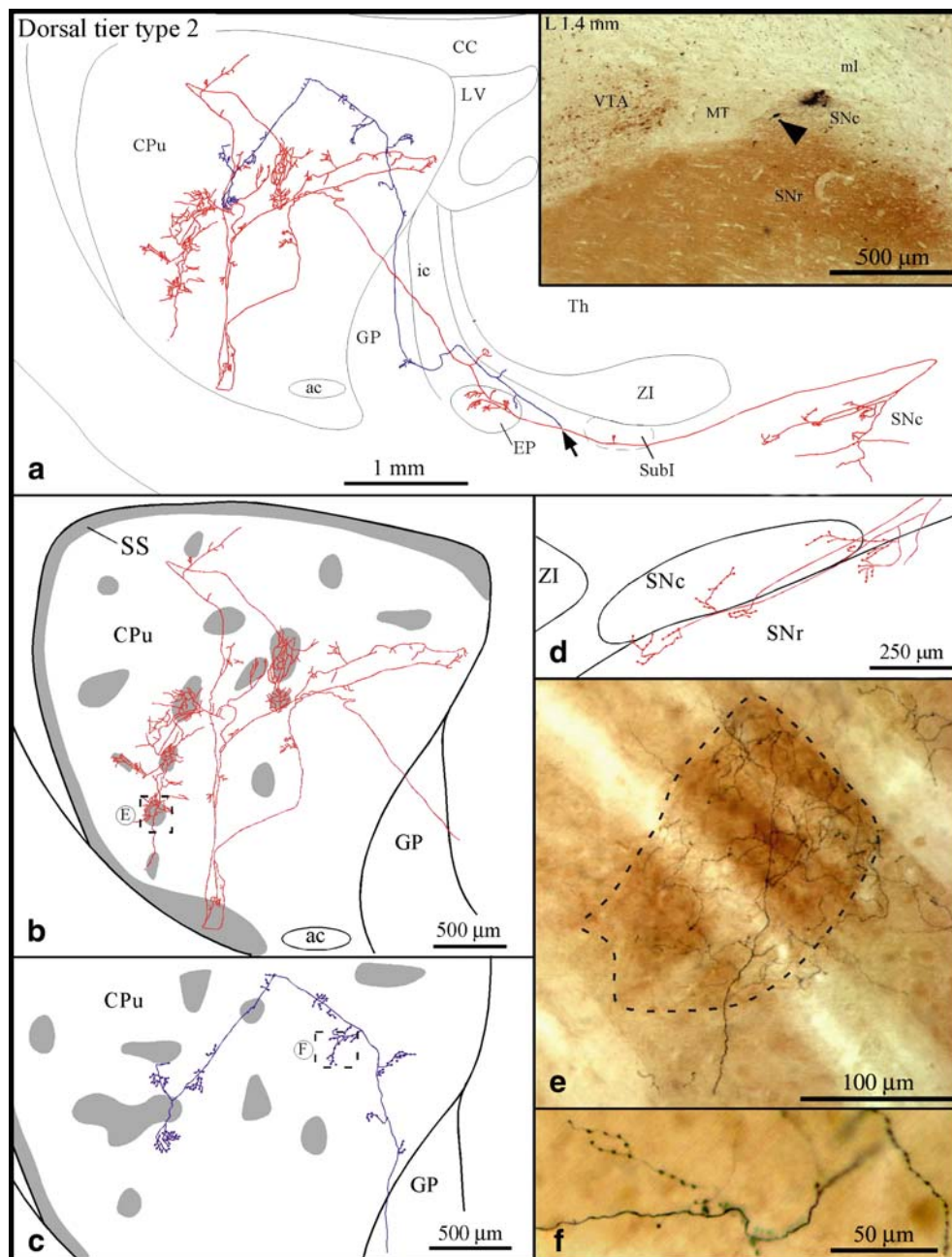


Fig. 5 (a) Camera lucida drawing of a dorsal tier type 2 SNc axon, the parent cell body of which is pointed out by the arrowhead in the inset. This neuron is located in the medial aspect of the dorsal tier of the SNc. The arrow in the drawing indicates the level at which the main axon bifurcates and the two axonal branches are represented in red and blue, respectively. (b, c) High power views of the striatal arborization of each axonal branch. The shaded areas indicate the striosomes and subcallosal streak. (d) Camera lucida drawing showing the distribution of the local axonal collaterals in the substantia nigra. (e, f) Photographic enlargements of the terminal arborizations of each axonal branch at the level indicated by the dotted rectangles in (b) and (c). Observe that the axonal branch represented in red arborizes profusely within the striosome delimited by the dotted line in (e) while the axonal branch illustrated in blue innervates the matrix compartment. For abbreviations see abbreviations list. Figure slightly modified from Prensa and Parent 2001

tier SNc neurons deeply embedded in the SNr. Interestingly, these ventral tier cells are the most severely affected in PD, and therefore, the DA depletion is not likely to solely influence the basal ganglia. The striosome/matrix specificity is another important organizational feature of the nigrostriatal

system. The existence of a preferential innervation of the matrix compartment and the striosomes by the dorsal and ventral tier neurons, respectively, has been reconfirmed by studies undertaken during the last decade. There are, nevertheless, convincing data showing that the ventral tier SNc

neurons deeply embedded in the SNr target the matrix. This finding is congruent with the fact that striatal DA denervation in PD is massive and involves both striosomes and matrix.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 5

The Localization of Inhibitory Neurotransmitter Receptors on Dopaminergic Neurons of the Human Substantia Nigra

HJ Waldvogel, K Baer, and RLM Faull

Abstract The substantia nigra pars compacta (SNc) is comprised mainly of dopaminergic pigmented neurons arranged in groups, with a small population of nonpigmented neurons scattered among these groups. These different types of neurons possess GABA_A, GABA_B, and glycine receptors. The SNc-pigmented dopaminergic neurons have postsynaptic GABA_A receptors (GABA_AR) with a subunit configuration containing α_3 and γ_2 subunits, with a small population of pigmented neurons containing α_1 $\beta_{2,3}$ γ_2 subunits. GABA_B receptors comprised of R1 and R2 subunits and glycine receptors are also localized on pigmented neurons. In contrast, nonpigmented (mainly parvalbumin positive neurons) located in the SNc are morphologically and neurochemically similar to substantia nigra pars reticulata (SNr) neurons by showing immunoreactivity for parvalbumin and GABA_ARs containing immunoreactivity for α_1 , α_3 , $\beta_{2,3}$, and γ_2 subunits as well as GABA_B R1 and R2 subunits and glycine receptors. Thus, these two neuronal types of the SNc, either pigmented dopaminergic neurons or nonpigmented parvalbumin positive neurons, have similar GABA_B and glycine receptor combinations, but differ mainly in the subunit composition of the GABA_ARs located on their membranes. The different types of GABA_ARs suggest that GABAergic inputs to these neuronal types operate through GABA_ARs with different pharmacological and physiological profiles, whereas GABA_BR and glycine receptors of these cell types are likely to have similar properties.

Keywords GABA_A • GABA_B and glycine receptors • Substantia nigra pars compacta

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Abbreviations

GlyR	Glycine receptors
<i>GLRA1</i>	GlyR $\alpha 1$ gene
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata

Introduction

The substantia nigra (SN) belongs to a group of large subcortical nuclei, the basal ganglia, which are involved in motor and mood control. The SN has two subdivisions: the substantia nigra pars compacta (SNc) and the substantia nigra pars reticulata (SNr); the SNc can be further subdivided into dorsal, ventral, and lateral tiers (Halliday 2004; McRitchie et al. 1995). The SNc contains dopaminergic, pigmented neurons that project mainly to the striatum in contrast to those of the SNr, which are mainly GABAergic and project to the thalamus, superior colliculus, and brainstem regions (Faull and Mehler 1978; Smith et al. 1998). Within the region of the SNc, there are also a small population of nonpigmented neurons similar in morphology to SNr neurons that are scattered among the pigmented neurons (Halliday 2004) the identity of which is not clear. The dopaminergic neurons of the SNc have been intensely studied due to the death of these cells in Parkinson's disease through as-yet-unknown causes and their importance in dopamine signaling throughout the brain (Braak et al. 2003; Gibb 1992).

Gamma-aminobutyric acid_A receptors or GABA_A receptors (GABA_ARs) belong to the superfamily of ionotropic receptors that include glycine, glutamate, and acetylcholine receptors (Cascio 2002; Colquhoun and Sivilotti 2004; Moss and Smart 2001; Rajendra et al. 1997). GABA_ARs are heteropentameric chloride ion channels that facilitate fast-response, inhibitory neurotransmission in the mammalian central nervous system and are assembled into pentameric

combinations of subunits from a variety of different classes (α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , θ and π) (Burt 2003) localized mainly on postsynaptic membranes of inhibitory synapses in the mammalian brain. They are the most widespread inhibitory receptors in the central nervous system and are associated with the tubulin-linker protein gephyrin, which functions as a postsynaptic organizer molecule for major subtypes of GABA_AR (Moss and Smart 2001). Many major drugs (e.g. benzodiazepines) act primarily via GABA_ARs, and extensive research efforts have focused on the GABA_AR because of its major role in inhibition in the central nervous system (Mohler 2007). Dysfunction of GABA_AR responses plays a role in neurological conditions including anxiety, epilepsy, and schizophrenia. In addition, mutations in certain GABA_AR subunits are associated with familial forms of idiopathic epilepsy (Scheffer and Berkovic 2003). To date, most available data on the distribution of GABA_ARs in the SN are derived from studies in rodents, but there is also evidence that GABA_AR are highly expressed in the human SNc (Waldvogel et al. 2008a). The results from electrophysiological and pharmacological investigations in animals have revealed a great complexity in the GABAergic control of pars compacta neurons (Tepper et al. 2007; Tepper and Lee 2007), which suggests that a similar functional diversity also exists in the human SNc.

Glycine receptors (GlyR) are important inhibitory receptors in the central nervous system and are especially prominent in the brainstem and spinal cord (Altschuler et al. 1986; Alvarez et al. 1997). The GlyR is strychnine sensitive and involved in regulating inhibitory chloride influx through chloride channels to stabilize the resting potential of neurons. GlyRs form pentamers assembled from a range of subunits (currently α_{1-4} , and β subunits), (Grudzinska et al. 2005; Langosch et al. 1990). Defects in mammalian glycinergic neurotransmission can result in hyperekplexia (Andrew and Owen 1997; Bakker et al. 2006). In humans, missense and nonsense mutations in the GlyR $\alpha 1$ gene (*GLRA1*), GlyR β subunit, and the GlyT2 transporter are the major cause of this disorder, although mutations in the multifunctional protein gephyrin have also been reported (Rees et al. 1994, 2001, 2002, 2003, 2006; Shiang et al. 1993). Gephyrin is responsible for the clustering of both GABA_A and GlyR at inhibitory synapses (Fritschy et al. 2008; Moss and Smart 2001).

GABA_B receptors on the other hand belong to the family of metabotropic G-protein coupled receptors, which provide a variety of physiological effects in the CNS (Bowery et al. 2002). These receptors have been shown to be comprised of at least two major subunits GABA_BR1 and GABA_BR2, each containing seven transmembrane spanning domains (Kaupmann et al. 1998a, b). These two subunits are thought to join at a common coiled domain to form functional

receptors associated with potassium and calcium channels (Jones et al. 1998). Previous immunohistochemical studies have shown that GABA_BR1 and GABA_BR2 subunits are distributed throughout the human SNc (Waldvogel et al. 2004). In studies of the *postmortem* human brain, we have used antibodies directed against α_1 , α_2 , α_3 , $\beta_{2,3}$, and γ_2 subunits of the GABA_AR, R1 and R2 subunits of the GABA_B receptor, and GlyR and used immunohistochemical procedures to visualize these different inhibitory receptor subtypes in the human SNc at the regional and cellular levels using both light and confocal laser scanning microscopy to provide a better understanding of inhibitory mechanisms in the human SN.

Materials and Methods

Brain Tissue

The human brain tissue for our studies was obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland). The University of Auckland Human Participants Ethics Committee approved the protocols used in these studies and all the tissue was obtained with full consent of the families. Brain tissue was obtained from neurologically normal cases, ranging from 48 to 83 years, with no history of neurological disease and no evidence of neuropathology and had a *postmortem* interval between 5 and 23 h after death.

For the receptor autoradiographic studies, blocks of the basal ganglia were dissected out, snap-frozen on dry ice, and stored at -80°C for subsequent processing for autoradiographic ligand binding studies of the distribution of GABA_ARs (Faull and Villiger 1986; Faull and Villiger 1988; Waldvogel et al. 2008b). For the autoradiographic localization of benzodiazepine-GABA_AR binding the slide mounted sections were processed as previously described (Faull and Villiger 1986; Waldvogel et al. 1998). Briefly, GABA_ARs were labeled by incubating the cryostat sections in 50mM Tris-HCL (pH7.4) containing 1nM [^3H]flunitrazepam (82.8 Ci/mmol, New England Nuclear), which binds to the Type I and Type II benzodiazepine binding site on the GABA_AR, or [^3H]Ro 15-1788 (82.8 Ci/mmol, New England Nuclear), a GABA_AR antagonist with a high affinity for central Type I and Type II receptors. The sections were washed (2×1 min. in Tris-HCl buffer, with a final dip in ice cold distilled water) and dried under a stream of cold air. All procedures were carried out at 4°C . Nonspecific binding was determined by the incubation of slides in the presence of $1\mu\text{M}$ clonazepam. Once dry, the slides were brought to room temperature, taped into X-ray cassettes and apposed with [^3H]-sensitive Hyperfilm (Amersham), and exposed in the dark at 4°C for 6–12 weeks. The films were developed in

D19, washed, fixed, and dried. The autoradiograms were scanned and saved as TIFF images and were printed using procedures to yield photographic autoradiograms (Fig. 1a).

Immunohistochemical Procedures

For the immunohistochemical studies, the human brains were processed as previously described (Waldvogel et al. 2006). In brief, the human brains were fixed by perfusion through the basilar and internal carotid arteries, first with phosphate-buffered saline (PBS) with 1% sodium nitrite, followed by 15% formalin in 0.1M phosphate buffer, at pH 7.4. After perfusion, midbrain is one word blocks containing the SN were dissected out and kept in the same fixative for 24 h. The tissue blocks were cryoprotected in 20% sucrose and then in 30% sucrose in 0.1M phosphate buffer with 0.1% Na-azide for approximately 1 week in each solution. The blocks were sectioned on a freezing microtome at 50 μ m and the sections were stored at 4°C in PBS with 0.1% sodium azide (PBS-azide).

Primary Antibodies

The following antibodies were used on postmortem human brain sections to detect GABA_ARs or to identify various cell phenotypes in the SN. The monoclonal antibody bd24 directed against an extracellular epitope of the α_1 subunit of the GABA_AR and the monoclonal antibody bd17 against the β_2 and β_3 subunits of the GABA_AR (H. Mohler and J.-M. Fritschy, Institute of Pharmacology and Toxicology, University of Zurich, Switzerland; Chemicon, MAB339) (Benke et al. 1991; Schoch et al. 1985). These antibodies have been used in several previous immunohistochemical studies of the human brain (Houser et al. 1988; Loup et al. 1998; Waldvogel et al. 1999). Three polyclonal guinea pig antibodies directed against the α_2 , α_3 , or γ_2 subunit of the GABA_AR were used (H. Mohler and J.-M. Fritschy, Institute of Pharmacology and Toxicology, University of Zurich, Switzerland) (Benke et al. 1996; Benke et al. 1991; Fritschy and Mohler 1995). A polyclonal rabbit anti-GABA_AR α_3 subunit antiserum (Alomone Labs, Israel) was also used. To label the two subunits of the GABA_B receptor (GABA_BR1 and GABA_BR2), three different antibodies raised in sheep and guinea pig were used. These antibodies were directed against sequences common to both human and rat GABA_B receptor subunits. Two GABA_BR1 antibodies were used; the first was raised against a sequence common to both the GABA_BR1a and GABA_BR1b receptor isoforms and was raised in sheep (ShR1) (Billinton et al. 2000), and the other GABA_BR1 subunit was raised in guinea pig (GPR1)

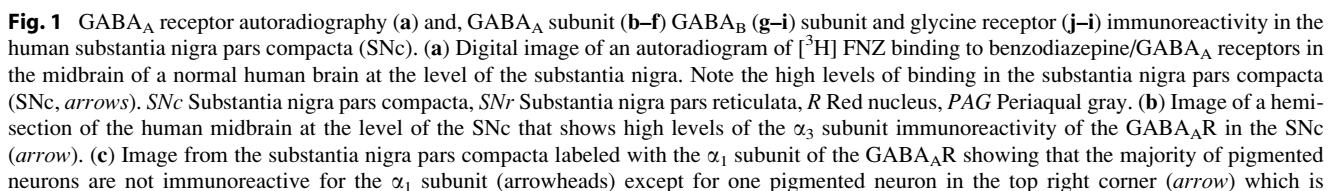
(AB1531, Chemicon Int. Temecula CA) against an extended overlapping sequence of the peptide used for the sheep antibody ShR1. The antibody against the GABA_BR2 subunit was raised in guinea pig (GPR2), (AB5394, Chemicon International. Temecula CA, USA).

Two antibodies were used to detect (GlyR) in the SNc: the monoclonal antibody Mab4a (Synaptic Systems; Germany), which recognizes the human GlyR α_1 subunit and also the 58 kDa GlyR β subunit; (Pfeiffer et al. 1984; Schroder et al. 1991) and a rabbit polyclonal antibody (RGlyR) raised against a peptide located in the N-terminus of the human GlyR α_1 subunit with cross reactivity to the GlyR α_2 . (Baer et al. 2003; Waldvogel et al. 1999, 2003, 2004, 2006, 2007a, 2008a). For cell type labeling rabbit anti-tyrosine hydroxylase (TH) (Protos-Biotech, NY, USA) and rabbit-anti parvalbumin (SWANT, Bellinzona, Switzerland) were used. All antibodies were dissolved in immunobuffer consisting of 1% goat serum in PBS with 0.2% Triton-X and 0.4% Thimerosol (Sigma).

Immunohistochemical Labelling

For light microscopic analysis, adjacent series of sections were selected and processed free-floating in tissue culture wells using standard immunohistochemical procedures (Waldvogel et al. 1999, 2004, 2007).

Sections were washed in PBS and 0.2% Triton-X (PBS-triton) and pretreated for antigen retrieval using standard protocols (Fritschy et al. 1998; Waldvogel et al. 2007a) before being processed for immunohistochemistry. Briefly, sections for antigen retrieval were transferred to six-well tissue culture plates and incubated in sodium citrate buffer solution, microwaved in a 650 W microwave oven for 30 s, and allowed to cool before washing (3 $\times$ 15 min) in PBS-triton. The sections were then incubated for 20 min in 50% methanol and 1% H₂O₂, washed (3 $\times$ 15 min) in PBS-triton, and incubated in primary antibodies for 2–3 days on a shaker at 4°C. The primary antibodies were washed off (3 $\times$ 15 min, PBS-triton) and the sections incubated overnight in species-specific biotinylated secondary antibodies. The secondary antibodies were washed off (3 $\times$ 15 min, PBS-triton) and the sections incubated for 4 h at room temperature in Extra-avidinTM, 1:1,000 (Sigma) or streptavidin peroxidase complex 1:1,000 (Chemicon). The sections were reacted in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% H₂O₂ in 0.1M phosphate buffer, at pH 7.4, for 15–30 min to produce a brown reaction product. Due to the neuromelanin pigment localized in pars compacta neurons having a similar colour to that of the DAB reaction product, other chromogen reactions were used, such as a nickel-intensified DAB procedure to produce different coloured reaction products such as a blue–black reaction



product (Adams 1981) and an alkaline phosphatase detection system was used to produce a blue reaction product. The sections were washed in PBS, mounted on gelatine chrom-alum coated slides, rinsed in distilled water, dehydrated through a graded alcohol series to xylene, and coverslipped. For double immunoperoxidase labeling, the primary and secondary antibodies were added sequentially with the first primary antibody reacted for nickel-intensified DAB to produce a black colour and the second primary reacted with DAB to produce a brown colour. For double immunofluorescence and confocal laser scanning microscopy, primary antibodies were bound to secondary antibodies conjugated with fluorochromes Alexa 488 and Alexa 594.

Control sections for single and double immunolabeling techniques were routinely carried out to determine nonspecific staining using the same immunohistochemical procedures as detailed earlier except that the primary antibodies were omitted from the procedure.

Results

Autoradiography

The autoradiograms resulting from [^3H] FNZ or [^3H] R0-15-1788 binding studies revealed a relatively high level of binding that correlated with the location of pigmented neurons in the SNc (Fig. 1a). This labeling was selectively higher in the SNc than that in the SNr on the same section.

Immunohistochemistry

The results showed that the various receptor subunits were present in a heterogeneous fashion on the pigmented and nonpigmented neurons in the human SNc. The pigmented neurons of the SNc were recognized by their red-brown cytoplasmic neuromelanin content. These neurons were

scattered in fragmented groups throughout the SNc to form dorsal, ventral, and lateral tiers (Halliday 2004) (Fig. 1b).

GABA_A Receptors (GABA_AR)

The various GABA_AR subunit antibodies produced a variety of distribution patterns and labeling intensities within the SNc. The most conspicuous GABA_AR subunit labeling in the SNc was the α_3 subunit, which was localized at relatively high levels throughout the SNc (Fig. 1b). At the cellular level, the α_3 subunit was localized mainly to the cell body and proximal dendrites of pigmented SNc neurons (Fig. 1d) and was also observed on dendrites of unknown origin traversing throughout the SNc. In addition, the α_3 subunit was also localized to small-diameter fiber bundles scattered throughout the ventral tier.

The two antibodies to the GABA_AR α_1 and $\beta_{2,3}$ subunits produced similar regional distribution patterns when viewed at low power on adjacent labeled sections. These subunits were largely absent from the pigmented neurons of the SNc except for a small proportion of pigmented neurons (2–7%) that had these subunits distributed over their cell body and dendritic tree (one example of an α_1 subunit positive neuron is shown in Fig. 1c, *arrow*). There were also small numbers of nonpigmented neurons with a morphology similar to that of SNr neurons that were intensely labeled with GABA_AR α_1 and $\beta_{2,3}$ subunits scattered among and between the pigmented neuronal groups (an example of a $\beta_{2,3}$ subunit labeled nonpigmented neuron is shown in Fig. 1e, *arrow*). Double labeling studies showed that these nonpigmented neurons were often labeled with parvalbumin. In addition, as detailed and illustrated (Fig. 2) in our recent previous publication Waldvogel et al (2008a), when examined more closely, different clusters of SNc pigmented neurons were associated with three different regional α_1 and $\beta_{2,3}$ subunit labeling patterns: (1) some groups of pigmented neurons had no cellular, dendritic or neuropil α_1 and $\beta_{2,3}$ subunit labeling associated with them except for a few intensely GABA_AR α_1

Fig. 1 (continued) immunoreactive for the α_1 subunit of the GABA_AR. There are also many long immunoreactive dendritic processes traversing the field of view. **(d)** Image of a pigmented neuron in the SNc immunolabeled with the α_3 subunit of the GABA_AR particularly on its cell body (*large arrowhead*) and proximal dendrites (*small arrowheads*). The other two out of focus pigmented neurons in the field show lower levels of labeling (*arrows*). **(e)** Section of the SNc labeled with $\beta_{2,3}$ subunit of the GABA_AR showing one $\beta_{2,3}$ subunit-IR non-pigmented neuron (*arrow*). Several immunoreactive dendritic processes are traversing the field of view. All of the pigmented neurons are not labeled for the $\beta_{2,3}$ subunits (e.g. *arrowhead*). **(f)** Pigmented neurons in the SNc immunoreactive for the γ_2 subunit of the GABA_AR particularly in the cytoplasm (*large arrowhead*) and on proximal dendrites (*small arrowhead*). **(g)** Image from the SNc showing GABA_BR1-IR labeling (black DAB-Ni) on the majority of pigmented neurons. (Identified by their brown coloured pigment). **(h)** Light micrograph of a GABA_BR2-IR section labeled with alkaline phosphatase to produce a blue reaction product on pigmented SNc neurons (*arrows*). **(i)** Confocal light microscopic image showing GABA_BR2-IR of a pars compacta pigmented neuron. Punctate receptor labeling can be identified along the proximal dendrites (*arrows*). **(j)** GlyR immunoreactivity of a section through the substantia nigra illustrating GlyR in the SNc on the various pigmented cell groups. Note the variations in the intensities of immunoreactivity in the different groups of pigmented neurons. **(k)** Pigmented neurons of the SNc that show GlyR immunoreactivity which is localised on cell bodies and dendritic processes (*arrow*). **(l)** High magnification image showing punctate GlyR labeling (*arrow*) on pigmented neurons of the SNc. Scale bars **a**, **b**=0.5 cm, **c**=50 μm , **d**–**i**, **k**, **l**=20 μm , **j**=250 μm

and $\beta_{2,3}$ subunit labeled nonpigmented (SNr-like) neurons scattered among the pigmented neurons, e.g. Fig 1c.; (2) some groups of pigmented neurons were associated with a high “background” or neuropil α_1 and $\beta_{2,3}$ subunit labeling with a few dendritic immunoreactive processes scattered throughout the region. Some of the neurons within this high neuropil group had low levels of GABA_AR α_1 and $\beta_{2,3}$ subunit immunoreactivity that weakly outlined their cell soma and dendrites; (3) The third group showed a medium to high neuropil label and high levels of cellular and dendritic GABA_AR α_1 and $\beta_{2,3}$ subunit immunoreactive nonpigmented neurons that were interspersed within the groups of pigmented neurons. To complement the immunoperoxidase labeling, double-immunofluorescent labeling was carried out using TH and parvalbumin to identify neuronal populations and α_1 and $\beta_{2,3}$ subunit antibodies to identify GABA_A receptors. SNc neurons were identified by TH staining and by the presence of pigment. Parvalbumin-labeled neurons were non-pigmented and scattered among the TH-positive neurons. This double labeling confirmed that the majority of TH positive neurons were generally not associated with α_1 and $\beta_{2,3}$ subunits but that these subunits were generally double-labeled with parvalbumin. Counts of pigmented neurons revealed that only a small subpopulation (6.5%) of pigmented neurons were outlined with α_1 subunit-IR, and 1.9% showed $\beta_{2,3}$ subunit-IR.

The α_2 subunit did not produce a reliable reproducible signal, although some brain sections did show a low level of labeling in the SNc.

The GABA_AR γ_2 subunit produced a pattern of labeling similar to that of GABA_AR α_3 subunit antibodies, and at the cellular level, showed an immunoreactive pattern similar to that of the α_3 subunit; that is, mainly in the cytoplasm and proximal dendrites (Fig 1f). The γ_2 subunit was also localized to the small-diameter fiber bundles similar to those seen with α_1 , α_3 , and $\beta_{2,3}$ subunit labeling and on the non-pigmented neurons labeled with α_1 , α_3 , and $\beta_{2,3}$ subunits.

GABA_B Receptors (GABA_BR)

Both pigmented and nonpigmented neurons in the SNc displayed immunoreactivity for both GABA_BR1 and GABA_BR2 subunits. Both sheep and guinea pig anti-GABA_BR1 subunit antibodies showed similar regional and cellular labeling patterns. The guinea pig GABA_BR2 subunit antibodies displayed a labeling pattern similar to that of the GABA_BR1. Virtually, all of the pigmented SNc neurons (95%) showed a black granular DAB-nickel reaction product in their soma and proximal dendrites for GABA_BR1 and GABA_BR2 subunits (Figs 1g–i). Both DAB-nickel and alkaline phosphatase were used as chromogens and these

produced either a black (Fig 1g) or a blue (Fig 1h) immunoreactive end-product respectively, which was clearly distinguishable from the red–brown neuromelanin pigment in the cytoplasm of the pars compacta neurons. Confocal microscopy also revealed punctate GABA_B staining on the cell surface of these neurons (Fig 1i, *arrows*). Double immunoperoxidase labeling showed that in contrast to the pigmented neurons a small number of GABA_B-IR nonpigmented neurons located in the pars compacta were immunoreactive for parvalbumin. All of these parvalbumin positive neurons located in and around the islands of pigmented pars compacta neurons (medial and ventral group) were immunoreactive for both GABA_BR1 and R2.

Glycine Receptors (GlyR)

The SNc contained high levels of GlyR immunoreactivity, although there were variations in the regional labeling patterns such that high levels of labeling was localized to various compacta cell groups, for instance, in the dorsal tier (*arrows* in Fig 1j), which is similar to the immunoreactivity observed for the GABA_AR α_3 subunit. At higher magnification, the GlyR immunoreactivity was observed scattered over the cell bodies and proximal dendrites of pigmented neurons as well as over nonpigmented neurons in the SNc (Fig 1k, l). Counts of immunoreactive neurons revealed that approximately 80% of the pigmented neurons had GlyR located on their cell bodies and proximal dendrites.

Discussion

From the immunohistochemical and autoradiographic data presented here, it is clear that several different receptor complexes are localized on neurons in the human SNc. GABA_AR α_1 subunit and $\beta_{2,3}$ subunits were present at low concentrations in the SNc, whereas α_3 and γ_2 subunits were more highly expressed. GABA_BRs were moderately expressed and GlyR subunits showed a moderate level of labeling compared with the very high levels observed in brainstem regions (Baer et al. 2003).

GABA_A Receptors (GABA_AR)

Binding studies carried out using [³H]Flunitrazepam, as illustrated in Fig 1a, resulted in a relatively high binding for this benzodiazepine ligand in the human SNc. This demonstrates binding to type I or type II GABA_A-benzodiazepine

receptors in the SNc. However, these binding studies do not have the cellular resolution to determine whether the binding is localized to the dopaminergic pars compacta cells. Very few studies have been published on GABA_AR binding in the human SNc, although several studies have described moderate binding in the SNr (Reisine et al. 1980; Speth et al. 1978). Animal studies confirm the presence of FNZ binding in the SNc of rats and jerbils (Olsen et al. 1985; Young and Kuhar 1980) and 6-OHDA lesioning of the SNc in rats demonstrated a reduced FNZ binding in the SNc (Nicholson et al. 1995), which indicates that the binding was localized to SNc neurons. Antidiazepam binding inhibitor (DBI) antibodies that recognize the β -carboline/benzodiazepine binding site of the GABA_AR were also localized to the human SNc (Ball et al. 1989) and staining with antibenzodiazepine antibodies produced moderate labeling of rat SNc neurons (Sanchez et al. 1991) further demonstrating the presence of benzodiazepine binding sites on SNc neurons.

Our immunohistochemical studies revealed two main types of receptors located on dopaminergic and nondopaminergic neurons of the human SNc. Immunohistochemical labeling revealed that the main GABA_AR subunits detected on pigmented neurons of the SNc are the GABA_AR α_3 and γ_2 subunits. The other receptor subtype containing mainly α_1 and $\beta_{2,3}$ subunits was found on parvalbumin-positive non-pigmented SNr-like neurons and also on a small subgroup of pigmented neurons present in the human SNc. The GABA_AR subunits detected in the human SNc agree with immunohistochemical studies in rats that determined that neurons in the SNc are immunoreactive for α_3 and γ_2 subunits and that the α_2 subunit was detected only at very low levels (Fritschy and Mohler 1995; Nicholson et al. 1992; Pirker et al. 2000; Rodriguez-Pallares et al. 2001; Schwarzer et al. 2001). The presence of a small subpopulation of α_1 and $\beta_{2,3}$ subunit TH-positive SNc neurons in the present study is supported by studies of the human and the rat SNc that also showed α_1 subunits on a subpopulation of TH-positive neurons (Ng and Yung 2000; Petri et al. 2002). Thus, there appears to be a small subset of SNc neurons in different mammalian species that show α_1 subunit immunoreactivity.

It is most likely that this small group of pigmented neurons also has α_3 and γ_2 subunits present and so have the full complement of subunits (α_1 , α_3 , $\beta_{2,3}$, γ_2) that the parvalbumin-positive SNr-like neurons of the SNc express. Double and triple labeling studies using antibodies to the various subunits, similar to our previous studies on pallidal neurons (Waldvogel et al. 1999), will be needed to clarify the subunit composition on individual neurons. Our double labeling studies of the human SNc also revealed that GABA_AR α_3 and γ_2 subunits in the human SNc are localized predominantly on neurons characterized neurochemically for tyrosine hydroxylase (TH) except for the small proportion (6.5%) of TH or pigmented neurons that were immunoreactive

for α_1 and $\beta_{2,3}$ subunits (Waldvogel et al. 2008a). In addition, the SNr-like neurons in the SNc that labeled for parvalbumin and contain mainly α_1 , α_3 , $\beta_{2,3}$, and γ_2 subunits may be displaced SNr neurons or may be equivalent to the group of GABAergic interneurons identified in the rat SNc (Hebb and Robertson 2000).

As has been established previously, the GABA_AR forms a pentameric subunit complex, and therefore it is most likely that other subunits may be present in the GABA_AR receptor complex on SNc neurons in addition to the α_3 and γ_2 subunits; For example, β_1 , α_4 , or δ subunits have been described in the rat SNc (Schwarzer et al. 2001) and the theta subunit was localized in the SNc in the primate brain (Bonnert et al. 1999).

The physiological role of GABAergic inhibition on SNc neurons has been investigated in animal studies. Electrophysiological experiments have revealed that the firing rates of dopaminergic neurons in the SNc are inhibited by the stimulation of the striatum, the globus pallidus, and the projection neurons of the SNr. The projections from the striatum and globus pallidus terminate on neurons of the SNr but also directly terminate on the dopaminergic neurons of the SNc (Bolam and Smith 1990; Celada et al. 1999; Smith et al. 1998). All of these nuclei are primarily composed of GABAergic projection neurons. The GABAergic neurons of the SNr are inhibited by projections from the striatum and the globus pallidus acting principally through GABA_ARs with the α_1 , $\beta_{2,3}$, γ_2 subunit configuration. Stimulation of these basal ganglia pathways or direct application of GABA leads to a burst firing pattern of SNc neurons. This inhibition has been shown to be mediated through GABA_AR located on dopaminergic neurons (Albers et al. 1999; Lavoute et al. 2007; Tepper and Lee 2007; Wedzony et al. 2001). Previous studies have also determined that it is principally the local axon collaterals from the SNr that appear to provide the highest inhibitory affect on SNc neuron firing rates (Paladini et al. 1999a). In addition, the release of GABA at synapses is important, but the specific pharmacological profile of the postsynaptic GABA_AR can influence the firing rates of dopaminergic SNc neurons. In this regard, it has been shown with recombinant receptor studies that GABA_AR containing either the α_1 or α_3 subunits display different pharmacological profiles; α_1 displays type I and α_3 displays type II pharmacology (Luddens et al. 1995). Therefore, for instance, the actions of GABA would be different on the majority of compacta neurons, which have receptors expressing mainly α_3 subunits than those on the subset of dopaminergic neurons expressing receptors containing α_1 subunits. The GABAergic striato-nigral pathway, which targets mainly the SNr neurons would also produce different responses in SNr neurons compared with SNc neurons. The influence that SNr neurons have on firing rates of the SNc neurons via GABA_AR would in turn affect dopamine release in the large

number of projection areas of the SNc, for instance, the striatum, the amygdala, the reticular nucleus of the thalamus, and the cerebral cortex (Anaya-Martinez et al. 2006; Fallon and Loughlin 1995).

Interestingly, electrophysiological experiments have indicated that dopamine release in the striatum can be activated differently by administering either muscimol (Celada et al. 1999) or gabazine in the SNc, and this can be interpreted as GABA acting via two different GABA_AR: those located on dopaminergic neurons or a type of interneuron located in the SNc (Balon et al. 2002b; Hebb and Robertson 2000; Lavoute et al. 2007). These interneurons were GAD positive, nondopaminergic, do not project to the striatum, and are thought to be locally projecting interneurons in the SNc. Therefore, the parvalbumin-positive neurons identified in our and other studies (McRitchie et al. 1996) in the human SNc may thus belong to this type of interneuron. On the other hand, they have a morphology similar to SNr neurons and may be displaced SNr neurons. Further studies are needed to clarify the role of these nondopaminergic neurons. Although the target of the subset of α_1 subunit containing SNc neurons is not clear, these neurons could form a unique negative feed back loop to the striatum as proposed by Petri et al. (2002). In addition, in general terms, the α_3 subunit is commonly associated with monoaminergic or cholinergic cellular subtypes (Fritschy and Mohler 1995; Rudolph and Mohler 2004) and has been implicated in muscle relaxant activity and thalamic oscillations, particularly via the reticular nucleus of the thalamus, which is a target of SNc neurons (Sohal et al. 2003).

Taken together with results in the rat, our studies would suggest that in the human SNc, GABA acts primarily via GABA_AR on dopaminergic neurons that have a subunit-specific configuration containing α_3, γ_2 subunits; in addition, GABA_A receptors are also found on a small subgroup of dopaminergic neurons containing $\alpha_1, \beta_{2,3}$ subunits and a group of parvalbumin-positive interneurons or displaced SNr-like neurons with $\alpha_1, \alpha_3, \beta_{2,3}, \gamma_2$ subunits.

GABA_B Receptors

In the present study, neurons of the SNc showed intense immunoreactivity for both the GABA_BR1 and GABA_BR2 subunits. The GABA_BR1 localization agrees well with previous immunohistochemical and *in situ hybridization* studies of humans (Billinton et al. 2000, 2001) and rats (Charles et al. 2001; Ng et al. 2001; Ng and Yung 2001). It is now generally accepted that the GABA_BR1 subunit dimerizes with the GABA_BR2 subunit to form a functional GABA_B receptor (Bowery et al. 2002; Jones et al. 1998; White et al. 1998). Also, molecular biological studies indicate that the

R1 and R2 subunits are present separately in the cytoplasm before dimerizing and being transported to the surface membranes (Couve et al. 1998). The cytoplasmic labeling observed in neurons in the present human study using the GABA_BR1 and R2 subunit antibodies may therefore be labeling this cytoplasmic receptor component. The small numbers of receptors seen at the cell surface and in the neuropil using these antibodies may represent functional membrane receptors. Our immunohistochemical results therefore suggest that the GABA_BR1 and R2 subunits represent both synaptic and extrasynaptic GABA_B receptors on pigmented neurons of the SNc, presumably at post synaptic sites. However, although light and confocal immunohistochemical resolution of receptors at the cell surface is not sufficient to determine whether they are localized on either pre- and/or postsynaptic membranes, previous studies suggest both pre- and postsynaptic localization of GABA_BR including extrasynaptic localizations (Boyes and Bolam 2007).

As already mentioned, the dopaminergic neurons of the SNc are innervated by GABAergic afferents from the globus pallidus, striatum, and SNr (Bolam and Smith 1990; Nitsch and Riesenberger 1988; Tepper et al. 1995). The effects of GABA_B receptors on GABA release onto dopaminergic neurons are thought to be mainly through presynaptic mechanisms by controlling the release of GABA from GABAergic afferents arising from the striatum, globus pallidus, and substantia nigra pars reticulata neurons (Giustizieri et al. 2005). In addition, GABA_BR also have an influence on the activity of SNc neurons and the release of dopamine (Balon et al. 2002a, b, c; Cobb and Abercrombie 2002; Paladini et al. 1999a, b; Saitoh et al. 2004; Santiago and Westerink 1992; Tepper et al. 1995). However the mechanisms of GABA_B actions are still unclear.

Glycine Receptors (GlyRs)

Relatively high levels of GlyR immunoreactivity were observed in the human SNc. On closer investigation, the GlyR immunoreactivity was localized to the main groups of pars compacta neurons and distributed on pigmented neurons and dendritic processes in the SNc. Counts of immunoreactive neurons revealed that approximately 80% of the pigmented neurons had glycine receptors located on their cell bodies and proximal dendrites.

The results of the present study in the SN are in general agreement with previous autoradiographic studies of the human brain (de Montis et al. 1982; Probst et al. 1986), which provide evidence that GlyR binding detected with [³H]strychnine was present in SNc. Although glycine terminals have been found in the SN of rats, the number of

terminals is relatively low (Rampon et al. 1996) and further studies are needed to determine the source and distribution of glycinergic terminals in the human SN. The pharmacological and physiological role of glycine in the SN is still unclear; however, studies in rats and cats have identified various effects of glycine in the SN including depression of dopamine neuron activity and reduced efflux of dopamine in the caudate nucleus (Cheramy et al. 1978; de Montis et al. 1982). In contrast, other studies demonstrated an increase of dopamine efflux in the SN with glycine application (Kerwin and Pycock 1979), as well as glycine-mediated effects of GABA transmission in the VTA region (Ye et al. 2004).

Inhibitory Receptors in Diseases Affecting the SN

The inhibition of neurons in the SNc is likely to be compromised in diseases of the basal ganglia such as Huntington's disease and Parkinson's disease. In Parkinson's disease, where the dopaminergic cells degenerate, the loss of dopamine in the projection fields will be affected, especially in the striatum. However, to date, large changes in GABA levels have not been found in the basal ganglia of Parkinson's patients, although GAD is increased in the indirect pathway and GABA is increased in the striatum (Kish et al. 1986). Also the levels of GABA_A and GABA_B receptors have been found to be affected in Parkinson's disease. Changes in GABA_A and GABA_B have been found in the GPi and the SNr in Parkinson's patients (Griffiths et al. 1990); however the results of these studies are variable (Galvan and Wichmann 2007). GABAergic dysfunction in Huntington's disease is also likely to affect the functioning of dopaminergic neurons in the SNc by creating an imbalance of GABAergic and dopaminergic effects in the striatum, which then leads to dysfunction in the basal ganglia circuitry. Further studies of the inhibitory system of the SNc in the human brain will provide a better understanding of the inhibitory mechanisms operating in the SNc and may possibly lead to better treatment strategies in diseases of the basal ganglia.

In summary, the inhibitory control of dopaminergic neurons is a complex mix of GABA_A, GABA_B, and glycine receptor modulation. These different receptor types are involved in both presynaptic release of GABA onto dopaminergic neurons (by GABA_BR) as well as postsynaptic activation via GABA_AR and GABA_BR. In addition, SNc neurons are also inhibited via the activation of glycine receptors located on dopaminergic neurons.

Due to the dopaminergic neurons of the SNc performing such an important role in the function of the basal ganglia and their key role in Parkinson's disease and other basal

ganglia disorders, further neuroanatomical studies of all of the inhibitory receptor types in both the SNc and in the SNr in the human brain will provide a better understanding of the complex inhibitory interactions that modulate the functioning of the SN.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 6

Basal Ganglia Control of Substantia Nigra Dopaminergic Neurons

Christian R Lee and James M Tepper

Abstract Although substantia nigra dopaminergic neurons are spontaneously active both in vivo and in vitro, this activity does not depend on afferent input as these neurons express an endogenous calcium-dependent oscillatory mechanism sufficient to drive action potential generation. However, afferents to these neurons, a large proportion of them GABAergic and arising from other nuclei in the basal ganglia, play a crucial role in modulating the activity of dopaminergic neurons. In the absence of afferent activity or when in brain slices, dopaminergic neurons fire in a very regular, pacemaker-like mode. Phasic activity in GABAergic, glutamatergic, and cholinergic inputs modulates the pacemaker activity into two other modes. The most common is a random firing pattern in which interspike intervals assume a Poisson-like distribution, and a less common pattern, often in response to a conditioned stimulus or a reward in which the neurons fire bursts of 2–8 spikes time-locked to the stimulus. Typically in vivo, all three firing patterns are observed, intermixed, in single nigrostriatal neurons varying over time. Although the precise mechanism(s) underlying the burst are currently the focus of intensive study, it is obvious that bursting must be triggered by afferent inputs.

Most of the afferents to substantia nigra pars compacta dopaminergic neurons comprise monosynaptic inputs from GABAergic projection neurons in the ipsilateral neostriatum, the globus pallidus, and the substantia nigra pars reticulata. A smaller fraction of the basal ganglia inputs, something less than 30%, are glutamatergic and arise principally from the ipsilateral subthalamic nucleus and pedunculopontine nucleus. The pedunculopontine nucleus also sends

a cholinergic input to nigral dopaminergic neurons. The GABAergic pars reticulata projection neurons also receive inputs from all of these sources, in some cases relaying them disynaptically to the dopaminergic neurons, thereby playing a particularly significant role in setting and/or modulating the firing pattern of the nigrostriatal neurons.

Keywords Basal ganglia • Dopamine neuron • Electrophysiology • Parkinson's disease • Substantia nigra

Abbreviations

<i>SK</i>	Calcium-activated potassium
<i>ChAT</i>	Choline acetyltransferase
<i>DA</i>	Dopamine
<i>EPSP</i>	Excitatory postsynaptic potential
<i>GPe</i>	External part of the globus pallidus
<i>GP</i>	Globus pallidus
<i>IPSP</i>	Inhibitory postsynaptic potential
<i>GPI</i>	Internal part of the globus pallidus
<i>M1</i>	Muscarinic receptor 1
<i>PD</i>	Parkinson's disease
<i>PPN</i>	Pedunculopontine nucleus
<i>SN</i>	Substantia nigra
<i>SNc</i>	Substantia nigra pars compacta
<i>SNr</i>	Substantia nigra pars reticulata
<i>STN</i>	Subthalamic nucleus
<i>VGLUT</i>	Vesicular glutamate transporter
<i>TRP</i>	Transient receptor potential

Introduction

The activity of substantia nigra pars compacta (SNc) dopaminergic neurons is influenced by the interactions between intrinsic membrane conductances and afferent input from other basal ganglia nuclei, as well as inputs from neurons outside the basal ganglia. When spontaneous activity is recorded in vitro where there is little afferent input, almost

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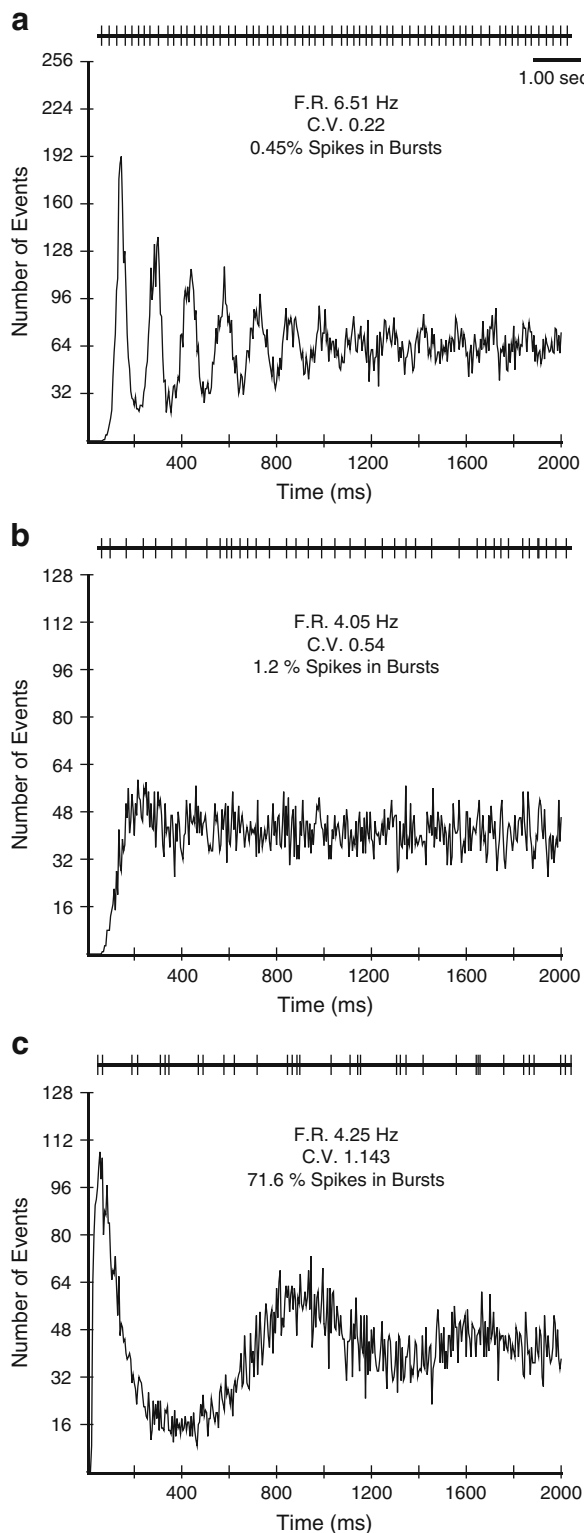


Fig. 1 Nigral dopaminergic neurons exhibit 3 distinct firing patterns or modes in vivo. (a) Pacemaker, (b) Random (c) Bursty. Each pattern gives rise to a characteristic autocorrelogram. Top insets show portions of the raw spike trains used to construct the autocorrelograms. Neurons exhibiting the pacemaker pattern with spikes occurring at fairly regular intervals produce autocorrelograms with three or more regularly occurring peaks (a). Neurons with spikes occurring more randomly produce

all nigral dopaminergic neurons exhibit a slow, very regular, pacemaker-like firing pattern (Grace and Onn 1989; Yung et al. 1991; Richards et al. 1997; Paladini et al. 1999b; Gulácsi et al. 2003). However, when dopaminergic neurons are recorded in vivo, it becomes clear that dopaminergic neurons exhibit a variety of different firing patterns (Bunney et al. 1973; Wilson et al. 1977; Grace and Bunney 1984; Freeman et al. 1985; Tepper et al. 1995; Hyland et al. 2002; Fà et al. 2003). The firing patterns of dopaminergic neurons can be seen as existing along a continuum but can be classified into one of three more or less discrete firing patterns, regular or pacemaker, irregular or random, and bursty, based upon the shape of the autocorrelograms as illustrated in Fig. 1 (Tepper et al. 1995). Single neurons may shift among these different patterns and many classes of drugs, in particular agonists or antagonists of the neurotransmitters contained in the principal nigral afferents, GABA, and glutamate, exert potent and stereotyped effects on the firing pattern of dopaminergic neurons (Overton and Clark 1992; Engberg et al. 1993; Tepper et al. 1995; Paladini and Tepper 1999; Prisco et al. 2002; Blythe et al. 2007). The mean firing rates of neurons exhibiting these different firing patterns can be equal, suggesting that the mechanisms responsible for controlling firing pattern are largely independent of those modulating the firing rate in nigral dopaminergic neurons (Wilson et al. 1977; Tepper et al. 1995; Paladini and Tepper 1999; Tepper and Lee 2007).

In addition, the discharge of action potentials by dopaminergic neurons recorded in vivo is only loosely correlated between neurons under most conditions, suggesting that the different firing patterns are modified, but not directly driven by afferent inputs (Hyland et al. 2002).

Functionally, changes in the firing rate and more importantly the firing pattern of dopaminergic neurons are translated into changes in dopamine levels in terminal regions, with the bursty firing pattern being most efficacious in increasing terminal dopamine levels, especially in the nigrostriatal pathway (Gonon and Buda 1985; Gonon 1988; Bean and Roth 1991; Manley et al. 1992; Chergui et al. 1994b; Lee et al. 2004; but see Floresco et al. 2003). Similarly, afferent input can affect the release of dopamine from the somatodendritic region of dopaminergic neurons (Chen and Rice 2002; Cobb and Abercrombie, 2002, 2003a), sometimes independently of striatal dopamine release (Trent and

autocorrelograms with an initial trough and a rise to a steady state (b) while neurons with many of their spikes occurring in bursts produce autocorrelograms with an initial peak which declines to steady state or a damped oscillation as in this case indicating rhythmic bursting (c). Note that the firing rates are largely similar between firing patterns while the coefficient of variation, defined as the standard deviation of the interspike interval divided by the mean interspike interval, exhibits a progressive increase from pacemaker to random and bursty neurons. FR Firing rate, CV Coefficient of variation

Tepper 1991; Cobb and Abercrombie 2003b), which could in turn modulate the strength of GABAergic input through pre-synaptic D₁ dopamine receptors as well as the firing of SNc dopaminergic neurons through D₂ autoreceptors (Cameron and Williams 1993; Seutin et al. 1994; Radnikow and Misgeld 1998; Misgeld et al. 2007). Thus, it is clear that an understanding of the afferent control of nigral dopaminergic neurons is an important prerequisite for understanding the complex interactions that take place both within the substantia nigra and throughout the basal ganglia network.

Afferent Inputs to SNc Dopaminergic Neurons

The basal ganglia are a collection of subcortical nuclei consisting of the neostriatum, the globus pallidus (GP), the subthalamic nucleus (STN), and the substantia nigra (Gerfen and Wilson 1996; Tepper et al. 2007), which is itself divided into the more dorsal pars compacta comprising primarily of dopaminergic neurons and the ventral substantia nigra pars reticulata (SNr) consisting primarily of GABAergic projection neurons (Lee and Tepper 2007b). Recently, some have argued that the pedunculopontine nucleus (PPN) should also be included as a basal ganglia nucleus (Mena-Segovia et al. 2004) and we include PPN afferents for the purposes of this review.

All of the basal ganglia nuclei project to the substantia nigra where they synapse on both dopaminergic and GABAergic neurons and most of the basal ganglia projections to the substantia nigra are GABAergic, with the exception of the projection from the STN, which is glutamatergic (Rinvik and Ottersen 1993) and the inputs from the PPN (Rye et al. 1987), some of which are glutamatergic and some of which are cholinergic (Futami et al. 1995; Takakusaki et al. 1996). In addition to the long-range projections from other basal ganglia nuclei, there is a significant inhibitory interaction between the GABAergic neurons in the SNr and the dopaminergic neurons in the SNc. As would be expected, the majority of the synapses formed on SNc dopaminergic neurons are GABAergic (Bolam and Smith 1990), although the majority of the afferents to dopaminergic neurons in the adjacent ventral tegmental area are not (Smith et al. 1996).

GABAergic Afferents

Neostriatum

The striatum is the principal input structure of the basal ganglia. Most striatal afferents are glutamatergic and excitatory and derive from the neocortex and intralaminar thalamic

nuclei (Kemp and Powell 1971; Ingham et al. 1998). Most of the corticostriatal and thalamostriatal inputs terminate in the spiny regions of the principal neuron, the striatal spiny projection neuron, which also forms the only output of the nucleus. Striatal spiny neurons project to the GP as well as to the dopaminergic neurons of the SNc and the GABAergic projection neurons of the SNr (Grofová and Rinvik 1970; Grofová 1975; Somogyi et al. 1981; Totterdell et al. 1984; Williams and Faull 1985; Bolam and Smith 1990; Bevan et al. 1994). Striatal projections to dopaminergic neurons terminate relatively distally. The striatonigral projection colocalizes substance P and dynorphin in addition to GABA and has been called the direct pathway, in contrast to the striopallidal projections to the external GP that colocalize enkephalin and are termed the indirect pathway (Gerfen and Wilson 1996). Substance P immunoreactive terminals form symmetric synapses on the dendritic shafts of SNc dopaminergic neurons, with only a small proportion of boutons synapsing on dopaminergic perikarya (Bolam and Smith 1990).

Globus Pallidus

The globus pallidus (external globus pallidus in higher mammals) sends inhibitory GABAergic projections to the STN as well as to both segments of the substantia nigra, thereby directly innervating both dopaminergic and GABAergic nigral neurons (Grofová 1975; Hattori et al. 1975; Totterdell et al. 1984; Smith and Bolam 1989, 1990; Smith et al. 1990; Bevan et al. 1996; Sato et al. 2000). Pallidal terminals form GABA-immunoreactive symmetric synapses that terminate on both the somata and proximal dendrites of nigral neurons, occasionally forming pericellular baskets around somata in the substantia nigra (Smith and Bolam 1990).

Substantia Nigra Pars Reticulata

The SNr provides one of the most important, yet least-understood and -characterized inhibitory inputs to nigral dopaminergic neurons. In addition to their long-range projections to the thalamus and the superior colliculus (Rinvik 1975; Clavier et al. 1976; Faull and Mehler 1978; Tokuno and Nakamura 1987; Harting et al. 1988; Kemel et al. 1988; Williams and Faull 1988; Bickford and Hall 1992; Deniau and Chevalier 1992; Redgrave et al. 1992; Mana and Chevalier 2001; Sidibé et al. 2002; Lee and Tepper 2007b), they also issue local axon collaterals that mediate the inhibition of neighboring dopaminergic and GABAergic neurons

within the substantia nigra (MacNeil et al. 1978; Walters and Lakoski 1978; Grace and Bunney, 1979, 1985a,b; Waszczak et al. 1980; Deniau et al. 1982; Hajós and Greenfield 1994; Häusser and Yung 1994; Tepper et al. 1995; Lee et al. 2004; Saitoh et al. 2004)

Local axon collaterals of SNr GABAergic projection neurons arborize in both SNr and SNc and exhibit considerable variability from neuron to neuron in terms of the size, extent of the collateral field, and its position with respect to the dendritic tree of the cell of origin, and frequently bear varicosities resembling both terminal and *en passant* boutons (Deniau et al. 1982; Grofová et al. 1982; Kemel et al. 1988; Nitsch and Riesenberger 1988; Tepper et al. 2003; Mailly et al. 2003; Lee and Tepper 2007b; Figs. 2 and 3). Electron microscopic analysis has revealed that the varicosities are large boutons that form symmetric synapses with somata as well as proximal dendrites, often forming multiple pericellular contacts (Damlama 1994; Tepper et al. 2003; Boyes 2004; Fig. 3) similar to those originating from GP axon terminals (Smith and Bolam 1990).

Glutamatergic Afferents

Subthalamic Nucleus

Although GABAergic afferents account for the majority of the basal ganglia inputs to nigral dopaminergic neurons, there are significant glutamatergic inputs as well. The best-characterized basal ganglia glutamatergic input to substantia nigra is from the STN (Hammond et al. 1978; Chang et al. 1984; Kita and Kitai 1987). Although injections of PHA-L into STN result in some labeling in SNc, the majority of labeled boutons are found in SNr. Subthalamonigral axons form boutons that contain small round vesicles and form asymmetric synapses on medium- and small-sized dendrites, mostly in SNr, and only rarely onto somata (Chang et al. 1984; Kita and Kitai 1987; Damlama 1994, Fig. 4). Most of these synapses are formed onto TH-immunonegative (presumably GABAergic) dendrites, with only about 10% of boutons originating from STN terminating on dopaminergic dendrites in SNr as shown in Fig. 4 (Damlama 1994).

Cholinergic Afferents

Pedunculopontine Nucleus

The projection from the PPN is neurochemically diverse and includes both glutamate and acetylcholine (Woolf and

Butcher 1986; Gould et al. 1989; Clements and Grant 1990; Charara et al. 1996). This is the only source of cholinergic input to nigral dopaminergic neurons. At least some PPN terminals express both choline acetyltransferase (ChAT) and the vesicular glutamate transporter (VGluT) and thus may be both cholinergic and glutamatergic (Lavoie and Parent 1994). The majority of boutons labeled from the PPN contain small round synaptic vesicles and form asymmetric synapses as shown in Fig. 4 and are glutamate immunoreactive, while a smaller proportion exhibits immunoreactivity for GABA and forms symmetric synapses (Charara et al. 1996). As with glutamate, GABA and acetylcholine appear to be colocalized in some cell bodies in the PPN (Jia et al. 2003).

Cholinergic synapses can be found on dopaminergic perikarya and dendrites as well as on GABAergic neurons in the substantia nigra (Beninato and Spencer 1988; Martínez-Murillo et al. 1989; Bolam et al. 1991; Charara et al. 1996). Although the majority (65%) of boutons anterogradely labeled from the PPN synapse onto nondopaminergic neurons and dendrites, the 35% that do synapse onto dopaminergic dendrites is significantly greater than the proportion of terminals from the STN, of which only 10% synapse onto TH+ dendrites. Furthermore, the PPN boutons tend to form synapses onto larger diameter dendrites than the STN boutons (Damlama 1994), as illustrated in Fig. 4, perhaps suggesting that the PPN is a more potent source of direct excitation of dopaminergic neurons than the STN (see below).

Control of Nigral Dopaminergic Neurons by Afferent Input

The anatomical organization of the basal ganglia afferents to the substantia nigra, and the microcircuitry within the substantia nigra itself forms much of the basis necessary for understanding the effects observed in response to stimulation of afferents to SNc dopaminergic neurons on both short- and long-time scales. These responses are sometimes unexpected and in many cases suggest that an important part of the afferent input to SNc dopaminergic neurons is relayed and filtered through the axon collaterals of SNr GABAergic neurons.

Responses to GABAergic Input

Striatum

Early studies showed that electrical stimulation of the striatum in vivo in cats produced a monosynaptic inhibitory

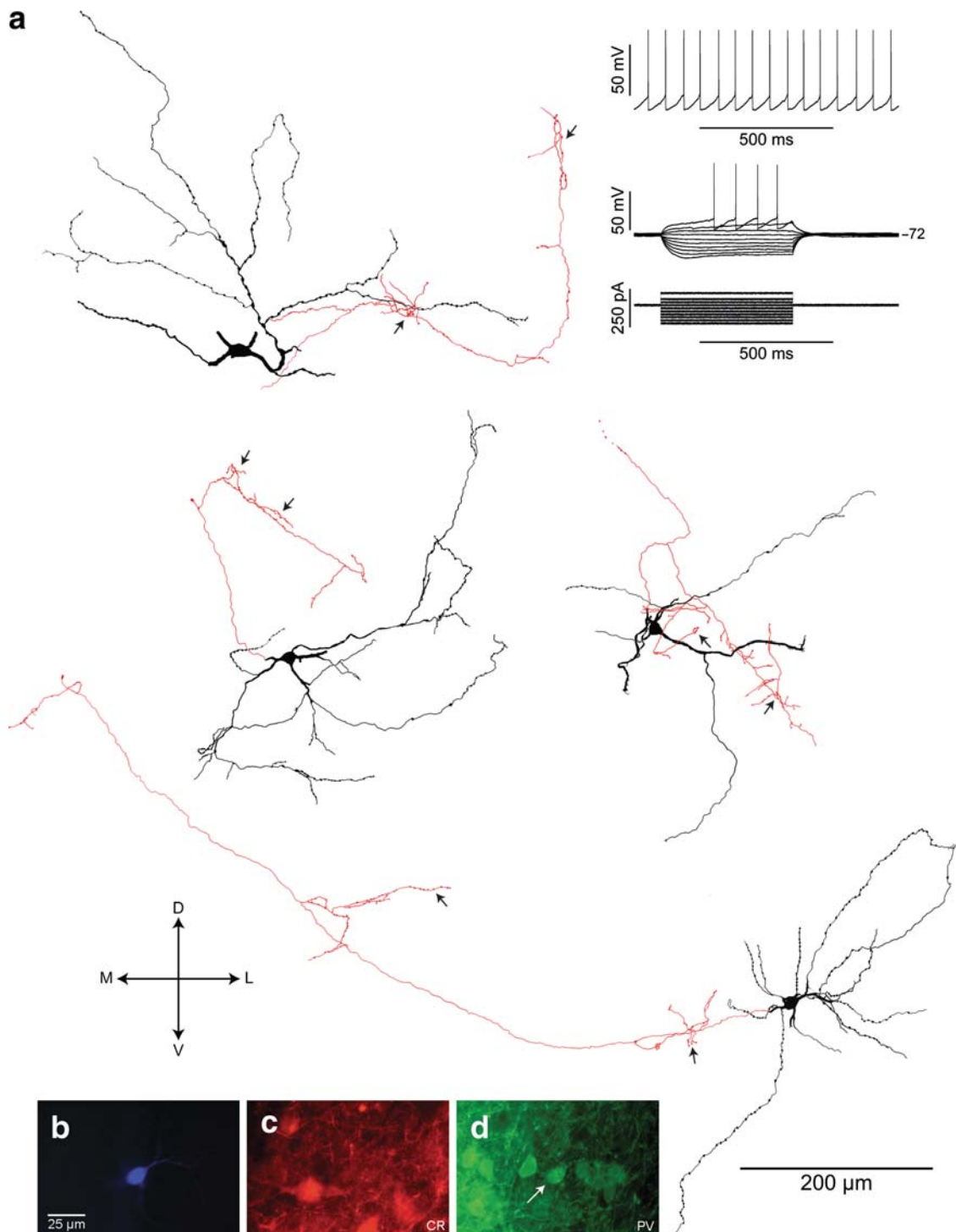


Fig. 2 Reconstructions of SNr GABAergic neurons filled with biocytin during whole-cell recording in vitro. (a) Representative examples of SNr GABAergic projection neurons recorded from coronal slices in vitro. Somata and dendrites are shown in black while axons are depicted in red. Note that all of the neurons issue local axon collaterals (black arrows) within the substantia nigra which in some cases can be observed to exhibit varicosities along their trajectories resembling *en passant* boutons as well as basket-like terminations with several large swellings characteristic of terminal boutons. Inset. Spontaneous activity and response to current injection (taken from bottom neuron) are typical for SNr GABAergic projection neurons. (b–d) Fluorescent images obtained from the bottom neuron show biocytin (b) calretinin (c) and parvalbumin (d). Note that the neuron exhibits immunoreactivity for parvalbumin (white arrow) but not calretinin. Other SNr GABAergic neurons containing calretinin as well as the small population containing both parvalbumin and calretinin similarly issue local axon collaterals. CR Calretinin, PV Parvalbumin, D Dorsal, V Ventral, M medial, L lateral. Orientation refers to reconstructed neurons. Modified from Lee and Tepper 2007b. Copyright 2007 Wiley-Liss, Inc

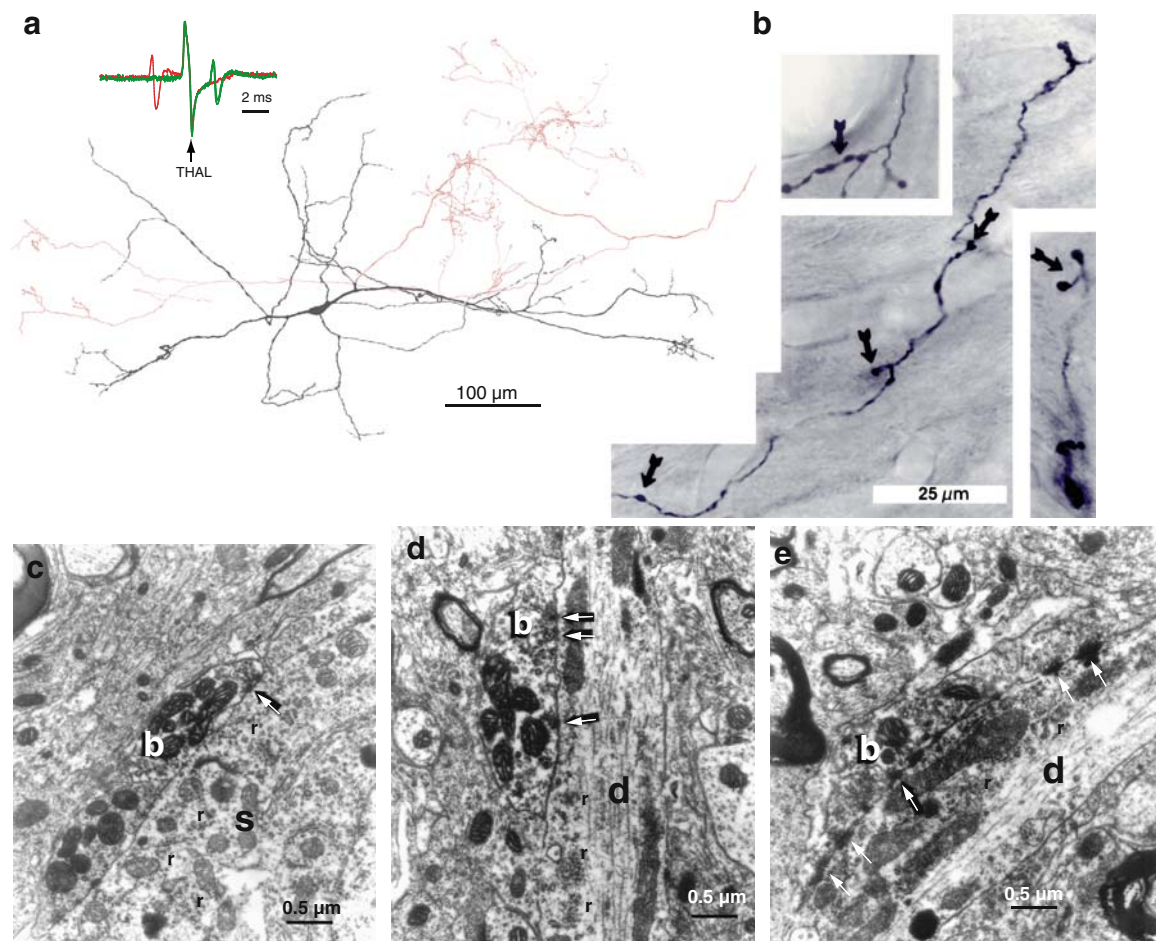


Fig. 3 Pars reticulata GABAergic projection neurons make synaptic contact with nigral dopaminergic neurons. (a) Reconstruction of an electrophysiologically identified rat nigrothalamic neuron juxtacellularly labeled with biocytin *in vivo*. The soma and dendrites are in black, the axon in red. Inset, 3 consecutive superimposed sweeps showing antidromic response of the nigrothalamic neuron following stimulation of the ventral thalamus (arrow). A collision is shown in the red trace. (b) High magnification light micrographs of portions of the local collateral arborization of a biocytin labeled nigrothalamic neuron. Note the varicosities (arrows) separated by long stretches of smooth axon. (c) Electron microscopic analysis of a biocytin filled varicosity shows that it is a large synaptic bouton (b) making a symmetric synapse (white arrow) onto the soma (s) of a dopaminergic neuron in pars compacta. Note the large number of free ribosomes (r) characteristic of dopaminergic neurons. (d) Large bouton (b) from a biocytin labeled nigrothalamic neuron makes a symmetric synapse onto a dopaminergic dendrite (d) in pars compacta. (e) Large biocytin-labeled bouton makes multiple symmetric contacts onto a large proximal dopaminergic dendrite in pars compacta

postsynaptic potential (IPSP) in unidentified nigral neurons that were almost certainly SNr GABAergic neurons (Precht and Yoshida 1971; Yoshida and Precht 1971). The IPSP had an onset latency of 14–20 ms and since an associated striatal-evoked field potential with the same latency was blocked by picrotoxin, this was considered to be a monosynaptic GABAergic response that we would today classify as being mediated by GABA_A receptors. Subsequent *in vivo* studies in rats recording from electrophysiologically identified dopaminergic neurons revealed similar monosynaptic inhibitory responses following striatal stimulation (Collingridge and Davies 1981; Grace and Bunney 1985a; Tepper et al. 1990; Paladini and Tepper 1999). This effect would be expected,

given the direct GABAergic projection from the striatum to SNc dopaminergic neurons (Bolam and Smith 1990).

However, when the stimulation intensity is decreased, SNc dopaminergic neurons respond with an increase in firing caused by the inhibition of SNr GABAergic neurons (Collingridge and Davies 1981; Grace and Bunney 1985a) that are more sensitive to GABAergic inhibition than dopaminergic neurons (Gulácsi et al. 2003; Fig. 5). Thus, under conditions of low to moderate levels of electrical stimulation, SNr GABAergic neurons are preferentially inhibited. The result is that SNc dopaminergic neurons are disinhibited from SNr GABAergic projection neurons and increase their firing rate (Grace and Bunney 1979, 1985a).

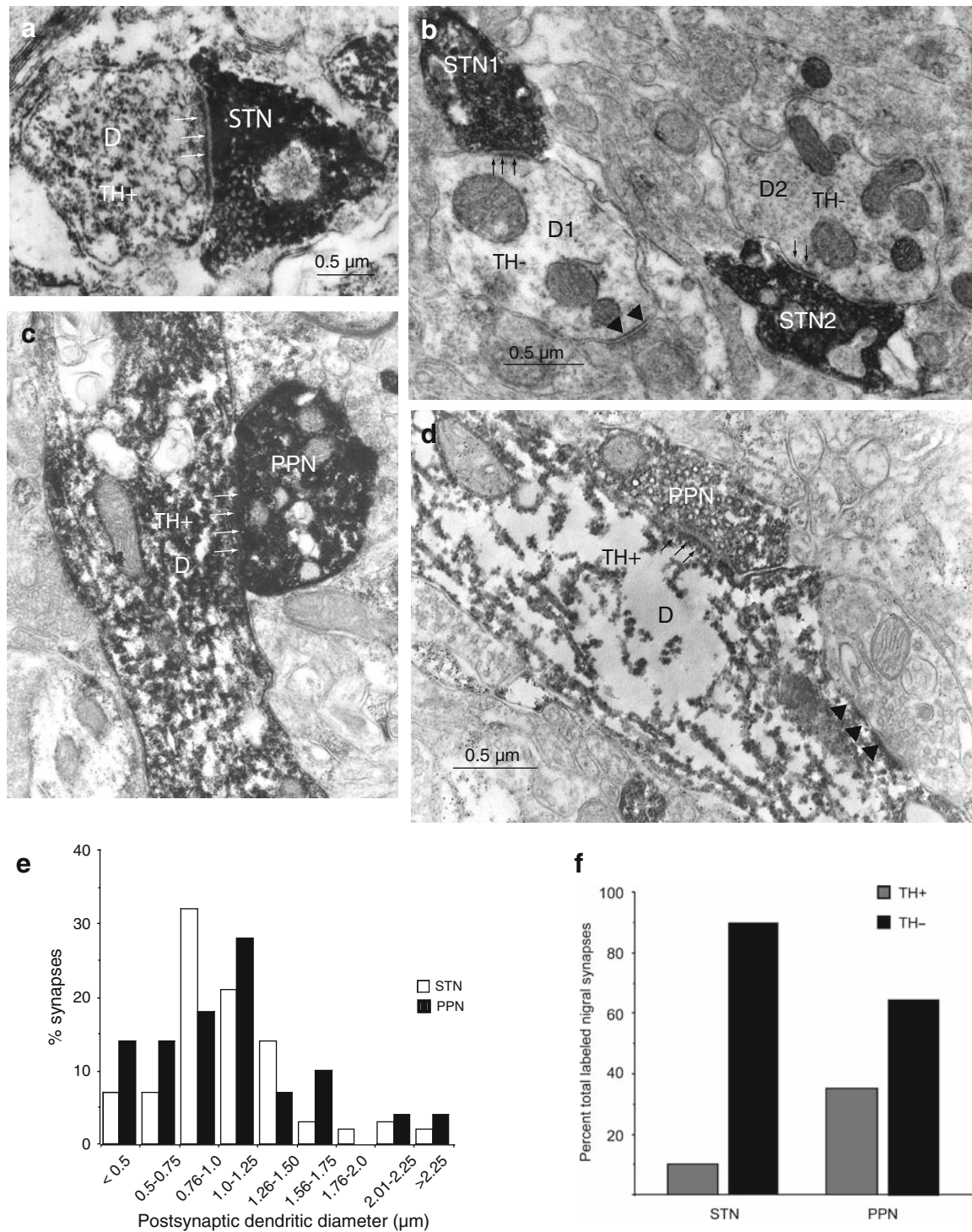


Fig. 4 Anterograde tracing of afferents from STN and PPN to substantia nigra. (a) A presynaptic bouton from STN labeled with PHA-L (STN) makes asymmetric synaptic contact (*arrows*) on TH-immunopositive (TH+) dopaminergic dendrite in pars compacta (D). (b) Two PHA-L labeled terminals from STN (STN1, STN2) make asymmetric synapses (*arrows*) onto medium-sized non-dopaminergic (TH-) dendrites (D₁, D₂) in pars reticulata. Note moderate postsynaptic thickenings that are much easier to see in the absence of TH immunolabeling. (c) Bouton (PPN) anterogradely labeled with PHA-L from PPN makes asymmetric synaptic contact (*arrows*) onto a medium sized dopaminergic (TH+) dendrite (D) in pars reticulata. (d) Another bouton labeled by PHA-L injection into the PPN makes a synapse onto a large proximal dopaminergic (TH+) dendrite in pars reticulata. (e) Histogram of the diameters of the dendrites at the site of synaptic contact made by afferents from STN and PPN showing that PPN afferents tend to make synapses with large, presumably more proximal dendrites than STN afferents. (f) Relative distribution of synaptic contacts in pars reticulata from STN and PPN afferents onto dopaminergic and non-dopaminergic dendrites. Note that the proportion of synapses made onto dopaminergic dendrites is much larger for boutons originating from PPN than from STN

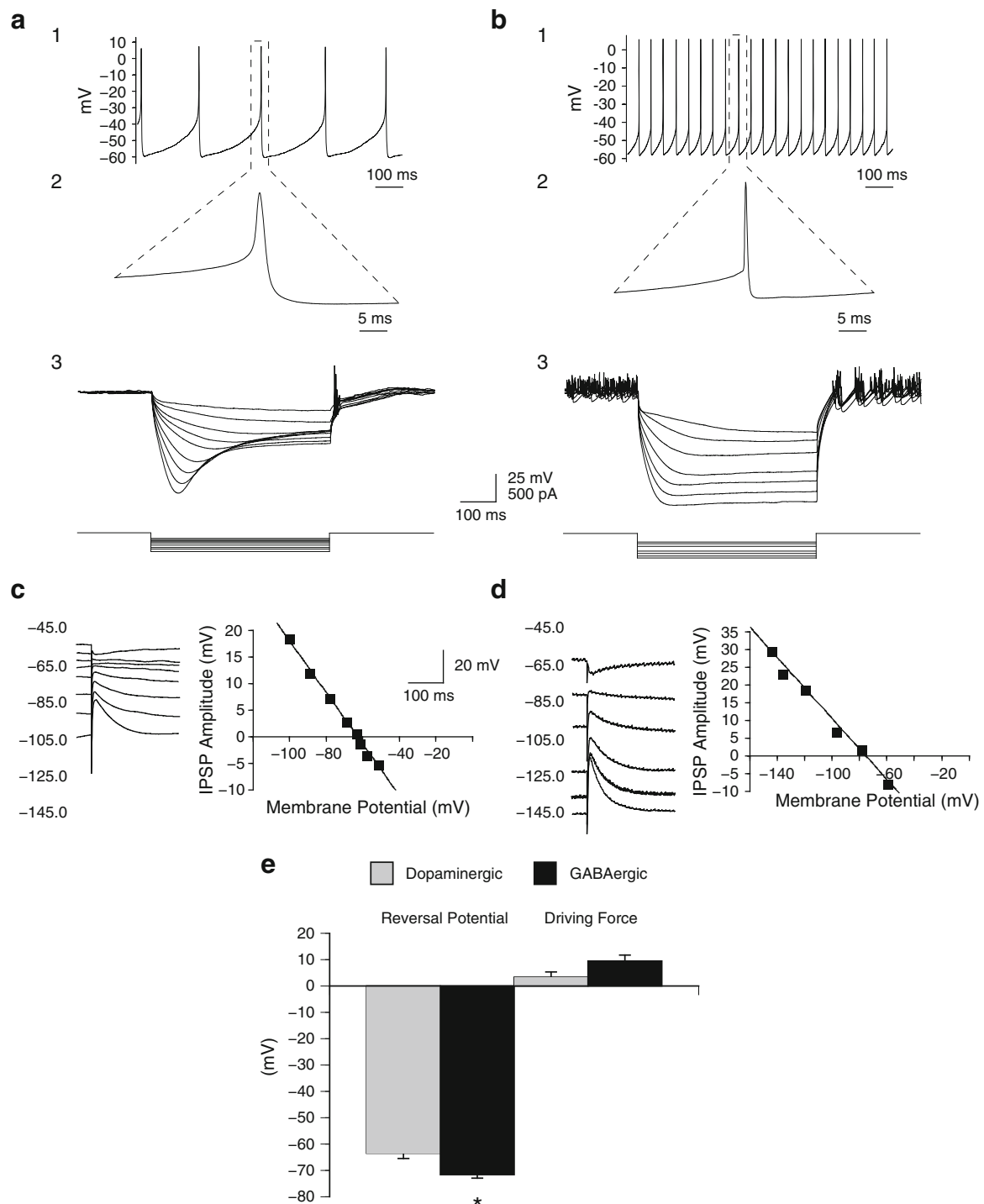


Fig. 5 The GABA_A IPSP is less hyperpolarizing in SNc dopaminergic neurons than in SNr GABAergic neurons. (**a1**, **b1**) Spontaneous activity of a substantia nigra dopaminergic (**a1**) and GABAergic (**b1**) neuron in vitro. (**a2**, **b2**) The action potential width and afterhyperpolarization duration is greater for the dopaminergic neuron (**a2**) compared to the GABAergic neuron (**b2**). (**a3**, **b3**) Responses to hyperpolarizing current pulses delivered from rest exhibit a strong sag caused by I_h in a dopaminergic neuron (**a3**) but not in a GABAergic neuron (**b3**). These characteristics allow for identification of nigral dopaminergic and GABAergic neurons based on their physiological properties in vitro. (**c**, **d**) Gramicidin perforated-patch recording of an SNc dopaminergic neuron (**c**) and SNr GABAergic neuron (**d**) showing the IPSP recorded at varying membrane potentials following local stimulation of the SNr. Reversal potentials were determined from plots of the IPSP amplitude against the membrane potential. (**e**) The GABA_A IPSP reversal potential was found to be less hyperpolarizing in dopaminergic neurons (-63.45 ± 2.02 mV) than in GABAergic neurons (-71.58 ± 1.37 mV). This corresponded to a greater hyperpolarizing driving force in GABAergic neurons (9.5 ± 2.2 mV) than in dopaminergic neurons (3.3 ± 2.0 mV). These findings indicate that nigral GABAergic neurons are more strongly hyperpolarized by GABA than the dopaminergic neurons, revealing an important mechanism underlying the seemingly paradoxical excitation of SNc dopaminergic neurons by GABAergic input and GABA_A agonists through SNr projection neurons. * $P < 0.01$. Modified from Gulácsi et al. 2003. Copyright 2003 the Society for Neuroscience

Globus Pallidus

Unlike afferents from the neostriatum the projection neurons of which fire slowly and episodically *in vivo* (Wilson 1993), the GP exerts a tonic inhibitory influence over SNc dopaminergic and SNr GABAergic neurons. Pallidal projection neurons fire spontaneously *in vitro* and exhibit very high spontaneous firing rates of ~10–100 Hz *in vivo* (DeLong 1971; Fillion and Tremblay 1991; Nambu and Llinas 1994; Celada et al. 1999; Cooper and Stanford 2000). The tonic inhibitory input generated by these high firing rates regularizes the firing pattern of SNc dopaminergic neurons *in vivo*. Electrical stimulation of the GP inhibits SNc dopaminergic neuron firing consistent with the monosynaptic innervation of SNc dopaminergic neurons by the GP (Paladini et al. 1999a). However, increasing pallidal neuronal activity by the local application of GABA_A receptor antagonists paradoxically *increases* the number of SNc dopaminergic neurons exhibiting the bursty firing

pattern and causes neurons exhibiting the random and pacemaker firing patterns to shift to the bursty firing pattern, rather than causing the expected inhibitory effects (Celada et al. 1999; Lee et al. 2004; Fig. 6). Functionally, the increase in burst firing caused by pallidal excitation leads to an increase in striatal dopamine levels (Lee et al. 2004; Fig. 6).

This is the same response that is observed when GABA_A receptor antagonists are infused locally within the substantia nigra, suggesting that the chemical stimulation of the GP leads to a reduction in inhibitory drive to SNc dopaminergic neurons. The explanation for this seemingly paradoxical response is that while the chemical stimulation of the GP results in an asynchronous release of GABA in substantia nigra, electrical stimulation causes a synchronous release. Although the asynchronous release is sufficient to effectively inhibit the more sensitive SNr GABAergic projection neurons, it does not effectively inhibit the less sensitive dopaminergic neurons with the overall result being a GABAergic disinhibition. The electrical stimulus, however,

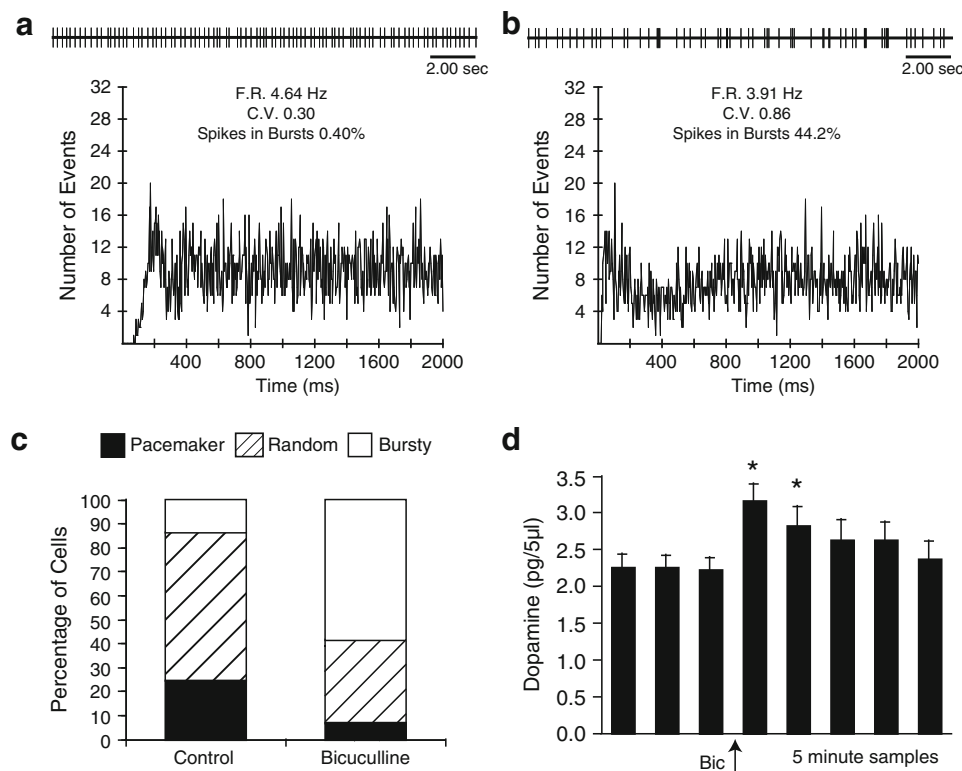


Fig. 6 Effects of pallidal excitation on SNc dopaminergic neuron firing pattern and striatal dopamine levels. **(a, b)** Autocorrelograms constructed from an extracellular recording of an SNc dopaminergic neuron *in vivo*. Portions of the raw spike train are shown above. This neuron was observed to exhibit a random firing pattern **(a)** which shifted to a bursty firing pattern following infusion of the GABA_A receptor antagonist, bicuculline into the GP **(b)**. Note that the firing rate (FR) was largely unchanged despite the significant increases in the coefficient of variation (CV) and overall percentage of total spikes fired in bursts. **(c)** The distribution of firing patterns exhibited by SNc dopaminergic neurons consisted mostly of the random firing pattern under control conditions, but pharmacological excitation of the GP with bicuculline shifted the distribution to one where the bursty firing pattern was most common. This is opposite to the effect that would be observed in response to a monosynaptic effect of the GP on SNc, demonstrating the important role of SNr GABAergic neurons in integrating synaptic input to the substantia nigra. **(d)** Simultaneous measurement of striatal dopamine levels with microdialysis revealed that pallidal excitation with bicuculline (arrow, Bic) led to a significant increase in striatal dopamine levels caused by the increase in burst firing. * $P < 0.05$. Modified from Lee et al. 2004. Copyright 2004 IBRO

causes a massive synchronous release that directly inhibits both SNr projection neurons and dopaminergic neurons with the overall result being direct inhibition of the dopaminergic neuron (Celada et al. 1999; Paladini et al. 1999a; Lee et al. 2004; Tepper and Lee 2007; Brazhnik et al. 2008).

In contrast to the dramatic changes observed in firing pattern, the local application of GABA_A antagonists or disinhibition caused by the chemical excitation of the GP produces less pronounced increases in the firing rate (Celada et al. 1999; Paladini and Tepper 1999; Lee et al. 2004). Conversely, inhibition of the GP results in a regularization of the firing of SNc dopaminergic neurons and a slight decrease in their firing rate as a result of reduced inhibition of SNr GABAergic neurons and the resultant increase in local inhibition (Celada et al. 1999).

Substantia Nigra Pars Reticulata

An important interaction between GABAergic SNr neurons and the overlying dopaminergic neurons was suggested by the finding of an inverse relationship between the spontaneous activity of some dopaminergic neurons and some pars reticulata nondopaminergic neurons in in vivo extracellular recordings (Grace and Bunney 1979). The study of this interaction has been complicated by the close proximity of the dopaminergic and GABAergic dendrites that are intermingled throughout SNr (Tepper et al. 1987). This precludes the direct stimulation of SNr GABAergic neurons and the recording of SNc dopaminergic neurons as has been used to study the afferent control exerted by other basal ganglia nuclei.

Although it has been postulated that intranigral inhibition might be carried out by specialized interneurons (Juraska et al. 1977; Francois et al. 1979; Grace and Bunney 1979, 1985a,b; Lacey et al. 1989; Johnson and North 1992; Bon-tempi and Sharp 1997; Hebb and Robertson 2000), the majority of the direct evidence suggests that the predominant sources of intranigral inhibition are the axon collaterals from SNr projection neurons (Deniau et al. 1982; Grofová et al. 1982; Tepper et al. 1995; Celada et al. 1999; Paladini et al. 1999a; Lee and Tepper 2007b; Brazhnik et al. 2008).

The GABAergic output neurons of the SNr exhibit spontaneous, pacemaker-like firing at high rates both in vivo (~20–40 Hz) and in vitro (~10–40 Hz) (DeLong 1971; Deniau et al. 1978; Guyenet and Aghajanian 1978; Nakanishi et al. 1987b; Lacey et al. 1989; Yung et al. 1991; Richards et al. 1997; Celada et al. 1999; Gulácsi et al. 2003; Windels and Kiyatkin 2004; Atherton and Bevan 2005; Lee and Tepper 2007b) and are thus well suited to serve as a source of tonic inhibition and the mediators of disinhibition of dopaminergic neurons.

Spontaneous GABA_A IPSPs are frequently encountered in SNc dopaminergic neurons in vitro, where afferent inhibitory projections from sources outside of the substantia nigra are disrupted, and local stimulation of the SNr in vitro elicits evoked IPSPs in dopaminergic neurons (Hajós and Greenfield, 1993, 1994; Häusser and Yung 1994; Fiorillo and Williams 1998; Saitoh et al. 2004; Gulácsi et al. 2003). Although these results are suggestive of the local inhibition of SNc dopaminergic neurons by SNr neurons, they could also be due to stimulus-evoked or spontaneous release of GABA from terminals arising from the striatum or GP (e.g., Iribe et al. 1999). The most definitive physiological evidence for the direct inhibition of SNc dopaminergic neurons by SNr projection neurons comes from the antidromic activation of local SNr axon collaterals by stimulating the thalamus or the superior colliculus in vivo, which produces powerful short latency inhibition of SNc dopaminergic neurons that cannot be mediated by anything other than monosynaptic synaptic connections made by the local axon collaterals of SNr projection neurons (Tepper et al. 1995; Paladini et al. 1999a; Brazhnik et al. 2008).

As mentioned earlier, SNr GABAergic neurons exhibit apparently greater sensitivity to inhibition by GABA than nigral GABAergic output neurons. This is due to different chloride regulatory mechanisms in the two cell types. The SNr projection neurons express KCC2, the typical potassium-chloride cotransporter found in most mature CNS neurons (Farrant and Kaila 2007) that keeps the intracellular chloride concentration low enough so that GABA_A receptor stimulation results in a hyperpolarizing IPSP. Dopaminergic neurons, on the other hand, lack this cotransporter (although they do express a different, less efficient chloride exchanger) with the result that the opening of chloride channels by GABA_A receptor activation produces a significantly smaller hyperpolarization that is responsible, at least in part, for the decreased sensitivity to GABAergic inhibition relative to the SNr output neurons (Gulácsi et al. 2003).

The increased sensitivity of SNr neurons to GABA and the resultant effects on the physiology of SNc dopaminergic neurons is manifest in several ways. SNc dopaminergic neurons respond to GABA_A receptor agonists applied either locally in the SN or administered intravenously with an increase in firing (MacNeil et al. 1978; Walters and Lakoski 1978; Grace and Bunney 1979; Waszczak et al. 1980) concomitant to a decrease in the firing rate of SNr GABAergic neurons (MacNeil et al. 1978; Walters and Lakoski 1978; Grace and Bunney 1979; Waszczak et al. 1980). This unique property likely underlies, at least in part, the rewarding effects of many drugs with abuse potential that act as GABA_A agonists such as ethanol, benzodiazepines, and barbiturates (Ross et al. 1982; Mereu et al. 1984; Mereu

and Gessa 1985; Tepper and Lee 2007). Further, the local infusion of a GABA_A agonist within the substantia nigra results in *increased* striatal dopamine levels (Santiago and Westerink 1992), and dopaminergic neurons that lack μ opioid receptors are *excited* by μ agonists (Lacey et al. 1989; Johnson and North 1992). All of these seemingly paradoxical effects are likely to be caused by disinhibition mediated via the GABAergic SNr projection neurons.

Receptors Mediating GABAergic Inhibition of SNc Dopaminergic Neurons

Anatomical studies have demonstrated that SNc dopaminergic neurons possess both ionotropic GABA_A and G-protein coupled GABA_B receptors (Bowery et al. 1987; Nicholson et al. 1992; Charara et al. 2000; Boyes and Bolam 2003). GABA_A receptors mediate a hyperpolarizing conductance that is carried mostly by chloride (Gulácsi et al. 2003; Farrant and Kaila 2007), while GABA_B receptors activate a potassium conductance (Lacey et al. 1988). The activation of either GABA_A or GABA_B receptors produces hyperpolarization and/or suppression of firing in vitro and causes an additional regularization of firing pattern in vivo (Grace and Bunney 1979; Waszczak et al. 1980; Pinnock 1984; Lacey et al. 1988; Erhardt et al. 1998; Gulácsi et al. 2003).

However, the vast majority of the evidence from in vivo studies in rats has suggested that SNc dopaminergic neurons are subject to tonic suppression of burst firing and are phasically inhibited primarily through GABA_A as opposed to GABA_B receptors (Nakamura et al. 1979; Grace and Bunney 1985a; Tepper et al. 1995; Paladini and Tepper 1999). The inhibition produced by the stimulation of GABAergic afferents from the striatum, GP, or SNr in vivo was found to be blocked by GABA_A but not GABA_B receptor antagonists in rat (Paladini et al. 1999a). In fact, the local application of GABA_B antagonists *potentiates* evoked inhibition and leads to the regularization of the firing pattern, suggesting that presynaptic GABA_B autoreceptors, located on all the basal ganglia GABAergic nigral afferents, are tonically activated in vivo and suppress GABA release and reduce evoked inhibition of SNc dopaminergic neurons (Paladini et al. 1999a; Paladini and Tepper 1999; Boyes and Bolam 2003). Presynaptic GABA_B receptors likely also suppress excitatory input to SNc dopaminergic neurons (Wu et al. 1999).

However, local electrical stimulation in vitro and especially high-frequency train stimulation can elicit slow, long-lasting, GABA_B-mediated IPSP and currents in SNc dopaminergic neurons, suggesting that GABA_B receptor activation from synaptically released GABA does indeed occur, but for some reason is not detected in the in vivo experiments

(Johnson and North 1992; Cameron and Williams 1993; Hajós and Greenfield, 1993, 1994; Häusser and Yung 1994; Saitoh et al. 2004). This is likely caused by the extrasynaptic location of GABA_B receptors in relation to GABAergic synapses (Boyes and Bolam 2003). In this situation, GABA must overcome reuptake and diffuse from the synaptic cleft to activate the extrasynaptic receptors, as has been shown in the hippocampus (Scanziani 2000). In some cases, the rhythmic firing of GABAergic afferents can cause sufficient GABA release to overcome reuptake and activate the extrasynaptic GABA_B receptors (Scanziani 2000), but this has not been observed following train stimulation of GABAergic afferents to SNc dopaminergic neurons in rat (Paladini et al. 1999a). The local application of GABA_A but not GABA_B receptor antagonists increases striatal dopamine levels, further indicating a tonic regulation by GABA through GABA_A but not GABA_B receptors (Santiago and Westerink 1992).

In recent in vivo experiments in mice, however, the stimulation of the striatum, GP, or SNr was shown to reliably induce inhibition with an early component mediated by GABA_A receptors and a late protracted component mediated by GABA_B receptors, even in response to single-pulse stimulation (Brazhnik et al. 2008). The late, GABA_B-mediated response was lengthened following the inhibition of GABA reuptake, suggesting that GABA reuptake mechanisms help to reduce GABA_B receptor activation in vivo (Brazhnik et al. 2008). Interestingly, the application of a GABA_B receptor antagonist in mice led to a slight decrease in the spontaneous firing rate and a slight regularization of the firing pattern of SNc dopaminergic neurons just as it does in rats (Tepper et al. 1995; Paladini and Tepper 1999) in the same neurons in which a blockade of postsynaptic GABA_B receptors attenuated the late inhibition, as shown in Fig. 7. Thus, just as in rat, there appears to be a tonic presynaptic stimulation of GABA_B autoreceptors without the tonic activation of postsynaptic GABA_B receptors (Brazhnik et al. 2008). The appearance of the late, GABA_B-sensitive component in mice but not rats was attributed to the smaller size and greater packing density of neurons of the mouse brain resulting in similar stimuli evoking substantially greater GABA release that was able to escape reuptake and reach extrasynaptic GABA_B receptors.

Responses to Glutamatergic Input

Subthalamic Nucleus

Subthalamic nucleus neurons fire spontaneously both in vivo and in vitro at ~6–30 Hz (Nakanishi et al. 1987a; Bergman

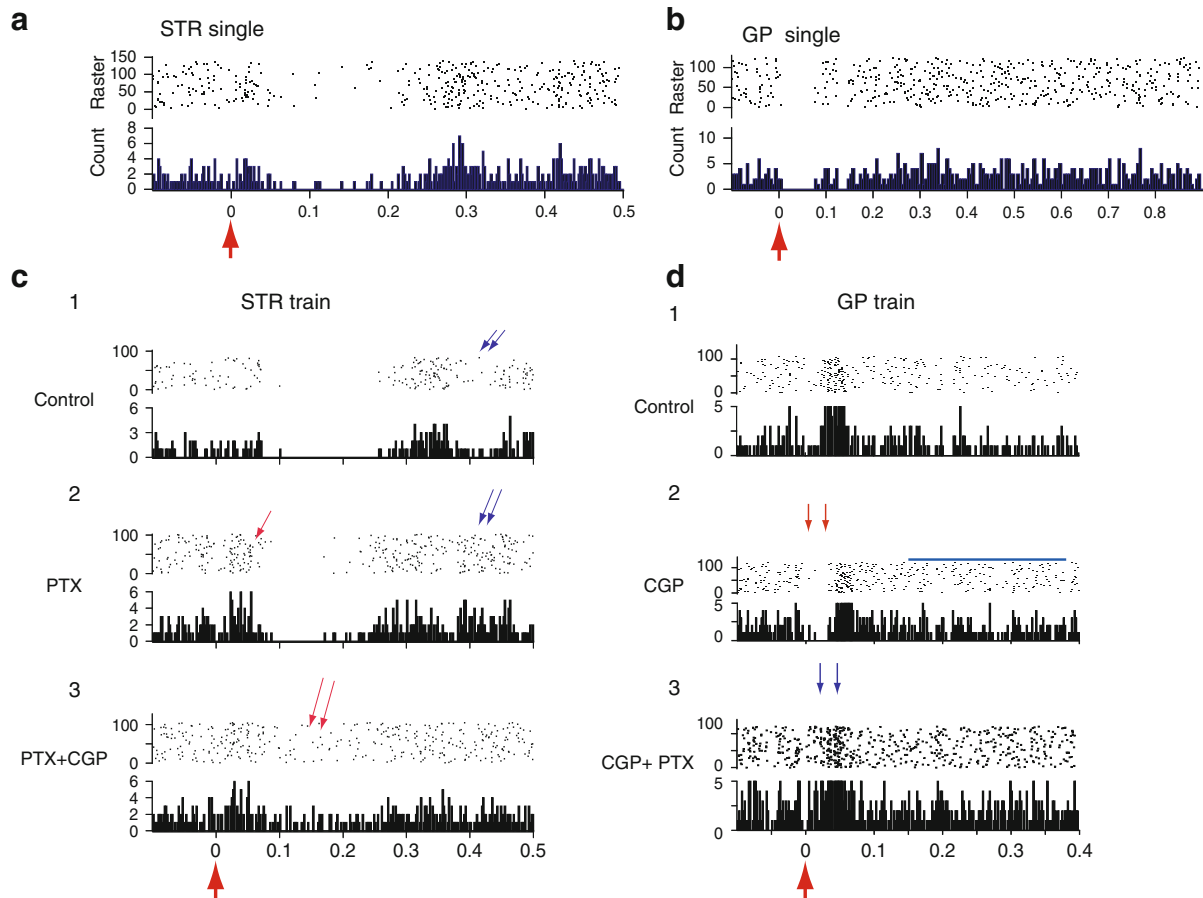


Fig. 7 GABAergic afferents to nigral dopaminergic neurons in mice exert both GABA_A and GABA_B receptor mediated effects. (a) Response of a substantia nigra dopaminergic neuron to single pulse stimulation of neostriatum (arrow). Note the rather long latency to the onset of the inhibitory response, the incomplete suppression of firing and the length of the inhibition that extends beyond 200 ms, all typical for striatal-evoked responses. (b) Similar stimulation of GP evokes an inhibitory response that exhibits a very short onset latency, a complete suppression of firing during the inhibition and shorter overall duration of inhibition than striatal-evoked responses (note different time scales in a and b). (c) The early and late components of the striatal-evoked inhibition of nigral dopaminergic neurons are mediated by different GABA receptors. (c1) Control recordings following trains of striatal stimulation (5 pulses of 300 μ A at 100 Hz). Note the second period of inhibition seen at around 450 ms (double blue arrows). (c2) Local pressure application of GABA_A receptor antagonist, picrotoxin (500 μ M) completely blocks the early part of the inhibition (unmasking an excitatory response as well, single red arrow) and the delayed inhibition (double blue arrows) but does not affect the late component of the inhibition. (c3) Subsequent simultaneous application of picrotoxin and the GABA_B-selective antagonist, CGP-55845A (500 μ M) blocks both components of the evoked inhibition indicating that the early inhibition is due to GABA_A receptor activation whereas the late inhibition is mediated by GABA_B receptor stimulation. (d) Both presynaptic and postsynaptic GABA_B effects can be seen in the same neuron. (d1) Control recordings of brief train stimuli delivered to GP at low intensity. (d2) Following application of CGP-55845A, the early inhibition is markedly strengthened due to increased GABA_A receptor activation (double red arrows) as a result of the blockade of inhibitory GABA_B autoreceptors on pallidonigral afferents. At the same time, CGP-55845A almost completely eliminates the late component of the inhibitory response (horizontal blue line) due to blockade of postsynaptic GABA_B receptors. D3. Subsequent simultaneous application of picrotoxin and CGP-55845A eliminates all inhibition. Modified from (Brazhnik et al. 2008). Copyright 2008 by the Society for Neuroscience

et al. 1994; Wichmann et al. 1994; Bevan and Wilson 1999; Beurrier et al. 1999; Do and Bean 2003; Hallworth et al. 2003; Wilson et al. 2006), thus providing SNc dopaminergic neurons with a source of tonic glutamatergic input.

Glutamatergic input, particularly via NMDA receptor stimulation, induces burst firing in vivo while blocking NMDA receptors in vivo leads to a regularization of firing pattern (Grace and Bunney 1984; Charlety et al 1991; Overton and Clark 1992, 1997; Chergui et al. 1993). Similar

results have been obtained in vitro (Johnson et al. 1992; Morikawa et al. 2003; Blythe et al. 2007). Lesions or pharmacological inhibition of the STN similarly decreases burst firing in SNc dopaminergic neurons (Smith and Grace 1992), most likely due to the decrease in NMDA receptor stimulation.

Burst firing can also be produced in vivo by the local blockade of GABA_A receptors. The local application of bicuculline or picrotoxin produces intense burst firing

(Tepper et al. 1995; Paladini and Tepper 1999; Brazhnik et al. 2008) as does the disinhibition of the GABAergic input from SNr (Celada et al. 1999; Lee et al. 2004). Either GABA_A or GABA_B receptor stimulation can prevent NMDA-induced burst firing in vivo or in vitro (Engberg et al. 1993; Seutin et al. 1994; Paladini et al. 1999b; Erhardt et al. 2002): an effect explained in computational studies by alterations in the dynamical interaction among membrane potential, conductance, and dendritic coupling (Canavier 1999; Komendantov et al. 2004; Kusnetsov et al. 2006). Thus, although glutamatergic input to NMDA receptors promotes burst firing, this effect of glutamatergic input on bursting in dopaminergic neurons is powerfully modulated by GABAergic afferents.

Experimentally induced increases in the activity of the STN by electrical or chemical stimulation have led to mixed effects on SNc dopaminergic neurons. Early in vivo recordings revealed a short latency excitation of SNc dopaminergic neurons elicited by STN stimulation (Hammond et al. 1978). Later experiments using either electrical or chemical stimulation of the STN in vivo revealed mixed excitatory and inhibitory responses, the latter being attributed to polysynaptic inhibition evoked by STN-induced excitation of pallidal or nigral GABAergic neurons (Robledo and Féger 1990; Féger and Robledo 1991; Smith and Grace 1992; Chergui et al. 1994a), with the initial short latency response consisting most often of inhibition (Smith and Grace 1992). However, longer duration pharmacological stimulation of the STN increased firing rate and induced burst firing in SNc dopaminergic neurons, which was at least partly due to the activation of NMDA receptors (Smith and Grace 1992; Chergui et al. 1994a). The explanation for these mixed effects was revealed to be a near simultaneous activation of a monosynaptic EPSP that was blocked by a non-NMDA receptor antagonist (although under certain conditions, an MK-801 sensitive component could be seen) and polysynaptic IPSP that was blocked by a GABA_A receptor antagonist. The mixed EPSP/IPSP survived transection of striatonigral and pallidonigral pathways indicating that the IPSP was due, at least in part, to STN-evoked activation of the axon collaterals of pars reticulata projection neurons (Iribe et al. 1999).

Furthermore, glutamatergic input can modify the firing pattern of SNr GABAergic neurons and evoke burst firing in those neurons as well (Ibáñez-Sandoval et al. 2007; Lee and Tepper 2007a). Therefore, the STN likely controls both the timing and pattern of inhibition coming from the SNr.

Although many of the effects of glutamate on dopaminergic neurons are mediated by NMDA receptors (Johnson and North 1992; Johnson et al. 1992; Overton and Clark 1992, 1997; Chergui et al. 1993; Meltzer et al. 1997b; Paladini et al. 1999b) while others are mediated by non-NMDA receptors (Zhang et al. 1994; Blythe et al. 2007), glutamatergic

input from the STN might affect SNc dopaminergic neurons through other mechanisms (Fiorillo and Williams 1998; Morikawa et al. 2003; Blythe et al. 2007). SNc dopaminergic neurons express both group I metabotropic glutamate receptors as well as ionotropic glutamate receptors (Martin et al. 1992; Ong et al. 1997; Paquet et al. 1997; Kosinski et al. 1998; Yung 1998; Chatha et al. 2000; Hubert et al. 2001; Kaneda et al. 2003). The actions of metabotropic glutamate receptors on the SNc dopaminergic neurons are complex. Group I receptor activation has been reported to cause an IPSP following brief agonist exposure in vitro that desensitizes following continued agonist exposure revealing an excitatory postsynaptic potential (EPSP) (Mercuri et al. 1993; Shen and Johnson 1997; Fiorillo and Williams 1998). The initial hyperpolarization is caused by calcium-activated potassium (SK) channel activation, while the depolarization is caused by activation of nonselective transient receptor potential (TRP) channels (Fiorillo and Williams 1998; Tozzi et al. 2003; Bengtson et al. 2004).

In vivo, and in vitro metabotropic glutamate receptor activation has been reported to potentiate burst firing and to exert mixed effects on the firing rate of SNc dopaminergic neurons (Meltzer et al. 1997a; Prisco et al. 2002). Similar to the GABA_B receptors, metabotropic glutamate receptors are localized at extrasynaptic sites, suggesting that reuptake might serve as a significant barrier to their activation (Hubert et al. 2001). In addition, group II and III metabotropic glutamate receptors have been reported to suppress excitatory synaptic input to SNc dopaminergic neurons (Wigmore and Lacey 1998; Valenti et al. 2005; Wang et al. 2005), suggesting that glutamate can affect the strength of afferent input which might act at ionotropic receptors.

Therefore, glutamatergic input to the substantia nigra can directly excite SNc dopaminergic neurons, indirectly inhibit them by exciting SNr GABAergic neurons, and directly inhibit or excite them by activating metabotropic glutamate receptors on the dopaminergic neurons.

Responses to Cholinergic Input

Pedunculopontine Nucleus

Although there is some heterogeneity in the physiological properties of PPN neurons and the identity of the neurotransmitters the neurons are releasing is unclear due to the high degree of colocalization of acetylcholine with glutamate, given that these neurons fire spontaneously at ~0.5–20 Hz in vivo and in vitro (Scarnati et al. 1987; Takakusaki et al. 1997), it seems reasonable to assume that SNc dopaminergic neurons receive tonic cholinergic and glutamatergic input

from the PPN. Consistent with this, the firing rate of SNc dopaminergic neurons decreases in response to the micro-infusion of exogenous acetylcholinesterase, which decreases cholinergic tone to SNc dopaminergic neurons (Greenfield et al. 1981).

Electrical stimulation of the PPN either in vitro or in vivo produces excitatory responses in SNc dopaminergic neurons. Excitatory postsynaptic potentials elicited by PPN stimulation in vitro are partly blocked by glutamate receptor antagonists and wholly blocked by the addition of acetylcholine receptor antagonists (Futami et al. 1995). In vivo electrical stimulation of the PPN produces short latency activation of most neurons recorded, which is also blocked by glutamate (non-NMDA) and acetylcholine receptor antagonists (Scarnati et al. 1986; Scarnati et al. 1987; Di Loreto et al. 1992; Lokwan et al. 1999). It is possible that the glutamatergic input to SNc dopaminergic neurons from the PPN favors the activation of non-NMDA receptors (Di Loreto et al. 1992; Meltzer et al. 1997b), but the basis for this is unclear. Nevertheless, the activity seen in SNc dopaminergic neurons following electrical stimulation of the PPN frequently contains burst firing (Lokwan et al. 1999). Chemical stimulation of the PPN results in an increase in burst firing, but not the firing rate of ventral tegmental area dopaminergic neurons, which are similar but not identical to nigral dopaminergic neurons in their responses to afferent input (Floresco et al. 2003; Keath et al. 2007).

Receptors Mediating Cholinergic Actions on SNc Dopaminergic Neurons

Anatomical studies have demonstrated the presence of both ionotropic nicotinic receptors and G-protein coupled muscarinic receptors in the substantia nigra (Deutch et al. 1987; Nastuk and Graybiel 1991). Nicotinic agonists potentiate glutamatergic EPSPs (Yamashita and Isa 2004) and the firing rate of nigral dopaminergic neurons decreases in response to a nicotinic antagonist (Clarke et al. 1985). The infusion of a muscarinic antagonist into the SN reduces striatal dopamine levels (Miller and Blaha 2005). Thus, both nicotinic and muscarinic receptors contribute to the tonic cholinergic modulation of SNc dopaminergic neuron activity. In addition, there are likely presynaptic effects mediated by the cholinergic input as acetylcholine acts to decrease glutamatergic and GABAergic input to midbrain dopaminergic neurons through muscarinic receptors (Grillner et al. 2000; Grillner and Mercuri 2002; Zheng and Johnson 2003) and enhances both glutamatergic and GABAergic inputs through nicotinic receptors (Mansvelder et al. 2002, but see Grillner and Mercuri 2002).

Peripherally administered nicotine induces an increase in the firing rate as well as burst firing in SNc dopaminergic neurons in vivo (Lichtensteiger et al. 1976, 1982; Clarke et al. 1985; Grenhoff et al. 1986). In vitro, nicotinic receptor stimulation causes a depolarization and increase in firing rate, but not burst firing, in midbrain dopaminergic neurons as well as the subsequent activation of a calcium-activated nonselective cation conductance (Calabresi et al. 1989; Pidoplichko et al. 1997; Sorenson et al. 1998; Yin and French 2000; Matsubayashi et al. 2003; Yamashita and Isa 2003).

Muscarinic receptor stimulation causes an increase in firing rate and burst firing in ventral tegmental area neurons in vivo, but only the effect on firing rate, not burst firing is observed in SNc dopaminergic neurons (Gronier and Rasmussen 1998). In vitro, muscarinic (M1) receptor stimulation causes a depolarization of SNc dopaminergic neurons and an increase in their spontaneous firing rate and the frequency of oscillatory potentials underlying firing (Lacey et al. 1990; Scroggs et al. 2001). However, with varying durations of activation, muscarinic receptors have been observed to cause hyperpolarization with brief activation and depolarization with more prolonged activation (Fiorillo and Williams 2000; Blythe et al. 2007). The initial hyperpolarization is caused by the activation of an SK channel, while the depolarization is likely caused by the activation of a nonselective cation current (Lacey et al. 1990; Fiorillo and Williams 2000). Nevertheless, the main effect of PPN stimulation is an increase in striatal dopamine levels, suggesting that the main role of the mixed input from the PPN is to increase activity in SNc dopaminergic neurons (Forster and Blaha 2003).

Summary and Conclusions

Though receiving input from outside the basal ganglia, the afferents from within the basal ganglia play a major role in the control and modulation of the firing rate and the pattern of activity of substantia nigra dopaminergic neurons. Although these neurons do not require any synaptic input to generate spontaneous activity, a tonic glutamatergic input from the STN and PPN increases their firing rate and appears necessary for burst firing. Burst firing is powerfully suppressed by GABAergic input originating from striatum, globus pallidus, and substantia nigra pars reticulata projection neurons and modulated by cholinergic input through both nicotinic and muscarinic receptors, as well as metabotropic glutamate receptors. Glutamatergic and cholinergic G-protein coupled receptors have been shown to attenuate synaptically evoked activity in SNc dopaminergic neurons in vitro, suggesting that brief, synaptically evoked activation

of these receptors is generally inhibitory, but different responses could be expected with varying durations of activation as might occur in vivo. Therefore, GABAergic, cholinergic, and metabotropic glutamate receptors largely act as gain control mechanisms, decreasing and increasing the effect of glutamate acting through ionotropic receptors on the firing pattern of SNc dopaminergic neurons.

The interactions of afferent input that shape the activity of SNc dopaminergic neurons are complex and involve a multitude of ionotropic and metabotropic receptors acting directly on the neurons themselves, as well as presynaptically shaping the inputs to them. In addition, the neurons can modulate the input they receive on a local level through dendritic dopamine release leading to the activation of presynaptic dopamine receptors that affect GABA release, as well as their own responsiveness to afferent input by controlling dendritic excitability through D_2 autoreceptors. The complex actions and interactions of afferent input to nigral dopaminergic neurons serve as the basis for the signaling repertoire displayed by these neurons, which through their effects on forebrain dopamine levels, influences much of the functioning of the basal ganglia as a whole.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 7

Substantia Nigra Control of Basal Ganglia Nuclei

Ezia Guatteo, Maria Letizia Cucchiaroni, and Nicola B. Mercuri

Abstract The substantia nigra, located in the ventral mesencephalon, is one of the five nuclei that constitute the basal ganglia circuit, which controls voluntary movements.

It is divided into the pars compacta and the pars reticulata, which mainly contain dopaminergic and GABAergic cells respectively. Here we overview the electrophysiological properties of these substantia nigra neurons in the pars compacta and reticulata, together with their synaptic connections, and discuss the functional effects of dopaminergic and GABAergic inputs within the basal ganglia. We also examine the phenomenon that when a deficiency of dopamine (DA) occurs (e.g. in Parkinson's disease), there is an aberrant synaptic plasticity in the basal ganglia.

Moreover, we point out that the appearance of an altered pattern of neuronal firing (beta-oscillations) and synchrony among neurons in the subthalamic nucleus, the internal globus pallidus, and the substantia nigra pars reticulata has been related to motor symptoms and possibly, persistent degeneration of DA-containing neurons.

Finally, we believe that, based on pathophysiological data, new and significant targets for therapeutic intervention can be identified and tested.

Keywords Basal ganglia • Dopamine neuron • Electrophysiology • Parkinson's disease • Substantia nigra

Abbreviations

DA	Dopamine
GP	Globus pallidus
GPi	Internal part of the globus pallidus
GPe	External part of the globus pallidus
SN	Substantia nigra
SNr	Substantia nigra pars reticulata
SNc	Substantia nigra pars compacta
STN	Subthalamic nucleus
SC	Superior colliculus
TANs	Tonically active interneurons
PD	Parkinson's disease
6-OHDA	6-hydroxydopamine
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
5-HT	Serotonin
NA	Noradrenaline
TTX	Tetrodotoxin
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
IP3	Inositol triphosphate
GIRK	G-protein linked potassium currents
SK	Small-conductance Ca^{2+} -dependent K^{+} channels
HCN	Hyperpolarization-activated cation current
HVA	High voltage-activated calcium channels
TRP	Transient receptor potential channels
IPSPs	Inhibitory postsynaptic potentials
REM	Rapid eye movements
FEF	Frontal eye fields

Organization of the Basal Ganglia Motor Circuit

The motor circuit is composed of several neuronal loops that, theoretically, originate from specific motor and premotor cortical regions, traverse the basal ganglia and the thalamus,

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and project back to the same areas of origin in the cortex (Alexander et al. 1986).

Thus, basal ganglia nuclei are components of the motor circuit that automatically controls the correct execution of voluntary movements. They consist of five interconnected entities: the caudate nucleus and putamen (both form the dorsal striatum), the globus pallidus (GP), the substantia nigra (SN) (pars compacta and reticulata), and the subthalamic nucleus (STN). The striatum and the STN represent the most important input stations receiving information from the cerebral cortex, whereas the internal part of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr) represent the major output nuclei, projecting to the motor thalamus and the brainstem. Two distinct pathways, one direct, monosynaptic and inhibitory (striatum-GPi / striatum-SNr), and the other indirect, polysynaptic and excitatory, that includes the external part of the globus pallidus (GPe) and the STN (striatum-GPe-STN) (Albin et al. 1989; Graybiel 1990), provide the synaptic communication between the striatum and the output nuclei (GPi and SNr, Fig. 1).

The output of the basal ganglia consists of GABAergic fibers (from GPi and SNr) that terminate in the thalamus providing an inhibition of the glutamatergic thalamo-cortical neurons. It is believed that the strength of thalamic inhibition depends on the net prevalence of the direct vs. the indirect pathway. In general, the activation of some striatal neurons is thought to inhibit GPi and SNr via the direct pathway, whereas the activation of different striatal neurons may have, via the indirect pathway, a net excitatory effect on GPi and SNr. As a consequence of this mutual modulation, an increased basal ganglia output would produce less movement through inhibition of the glutamatergic thalamo-

cortical neurons, whereas reduced basal ganglia output would result in an increased movement, due to disinhibition of the same thalamic neurons (reviewed by DeLong and Wichmann 2007).

Notably, an important internal loop, constituted by the nigro-striatal DAergic projection, provides the source of dopamine (DA) in the dorsal striatum.

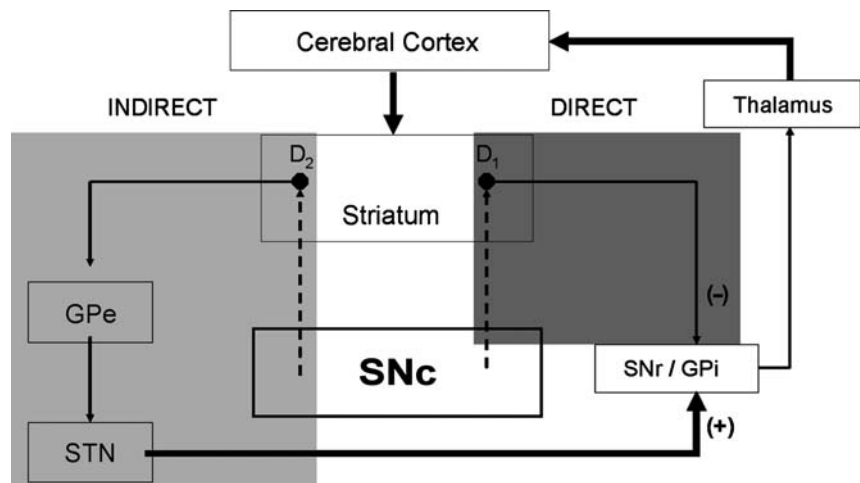
Undoubtedly, the SN, situated in the ventral mesencephalon, is an essential site regulating the overall execution of movements. It is divided into two functionally and anatomically distinct areas: the pars compacta, where the neurons synthesizing DA and projecting to the striatum are located, and the pars reticulata, where the GABAergic nigro-thalamic/nigro-tectal output neurons originate.

Here, we present data regarding the functional aspects of SN neurons, their connections, and the modalities by which they control neuronal activities in target areas.

Electrophysiological Properties of Substantia Nigra Compakta Daergic Neurons

The neurophysiological characteristics of DAergic neurons in the substantia nigra compacta (SNc) are typical so that they can be easily identified by electrophysiological techniques. These neurons have a broad action potential (>1.5 ms), which appears biphasic in extracellular recordings (Diana and Tepper 2002). During intracellular and patch-clamp recordings, the action potential is followed by an afterhyperpolarization that is mainly due to calcium-activated potassium conductances (Shepard and Bunney 1991). Another distinct feature of DAergic neurons is that they have a

Fig. 1 Model of basal ganglia circuit. The cortical motor areas are connected to a specific subcircuit (basal ganglia) in which the substantia nigra is an important site of regulation. Thick arrows indicate excitatory inputs; thin arrows, inhibitory inputs; dashed arrows, either inhibitory or excitatory projections. (+) indicates the net excitatory input of the indirect pathway onto the output nuclei; (−) indicates the net inhibitory input of the direct pathway onto the output nuclei. Abbreviations: *GPe* External globus pallidus, *GPi* Internal globus pallidus, *STN* Subthalamic nucleus, *SNc* Substantia nigra pars compacta, *SNr* Substantia nigra pars reticulata



regular spontaneous firing activity (1–3 Hz) when recorded in slices of the ventral mesencephalon (Grace and Onn 1989, also see Lee and Tepper in this issue).

The firing is mainly regulated by calcium currents, particularly L-type, which are very important in sustaining the depolarizing oscillations that regulate pacemaker activity (Mercuri et al. 1994) and by calcium-activated potassium conductances, located on both soma and dendrites (Wilson and Callaway 2000). These potassium channels are also activated by Ca^{2+} released from intracellular stores, as result of either metabotropic glutamate receptor activation (Fiorillo and Williams 1998) or spontaneous events (mainly in neonate rats) (Seutin et al. 2000). It is generally accepted that sodium currents act to amplify calcium-dependent oscillations but are not required for their generation. Moreover, a distinctive feature of DAergic cells is that they present, when hyperpolarized, a sag potential that is due to a prominent time- and voltage-dependent conductance (Ih, Fig 2b) (Mercuri et al. 1995).

In *in vivo* conditions, DAergic cells can either be silent or show regular, low-irregular, and bursting firing activity. Interestingly, pace-maker cellular activity regulates tonic release, while bursting activity promotes phasic DA release. This is very important in controlling voluntary motor performances (Grace 1988).

An additional phenomenon that modulates the firing of DAergic cells is the release of DA from the soma and the dendrites of these neurons. Thus, the mesencephalic release of DA activates D_2 autoreceptors located on the membrane of SNc neurons, providing an inhibition of firing via activation

of G-protein-linked potassium currents (GIRK, Fig 2d) (Lacey et al. 1987).

Synaptic Connections of the Substantia Nigra Pars Compacta Neurons

The DAergic cells (containing neuromelanin in primates) mainly project to the dorsal striatum, as well as to extra-striatal areas such as the STN and the GP (Lindvall and Bjorklund 1979; Cossette et al. 1999). Crucial information that derives from clinical/pathological studies is that they mainly coordinate the motor aspects of behavior. It is worth noting that, while the degeneration (hypoactivity) of the nigro-striatal DA system determines hypokinesia and rigidity (Parkinsonian symptoms), its hyperactivity could favor hyperkinetic movements (e.g. Chorea).

In general, the firing rate of SNc DA neurons is negatively modulated by GABAergic afferents (Fig 3). In fact, DAergic neurons receive GABAergic inputs from the striatum and the GP (Ribak et al. 1980; Smith and Bolam 1989), from the projecting neurons of the adjacent SNr, and from GABAergic interneurons (Tepper et al. 1995). Therefore, the GABAergic afferents are tonically activated, stimulating postsynaptic GABA_A and pre- and postsynaptic GABA_B receptors.

On the contrary, a strong excitatory input, mediating burst firing in DAergic neurons, derives from the glutamatergic projections of the medial prefrontal cortex, STN, pedunculo-

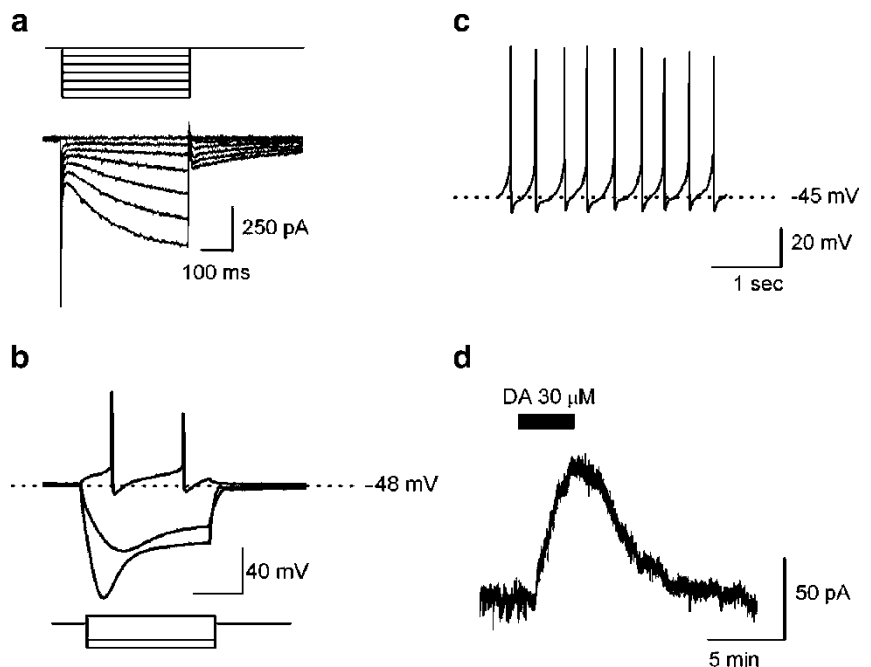


Fig. 2 Electrophysiological properties of DAergic SNc neurons recorded in *in vitro* rat slices. DAergic neurons express a time-dependent hyperpolarization-activated current (Ih) in response to hyperpolarizing voltage steps (a) In current-clamp recordings this current causes a “sag” potential (b) at hyperpolarized potentials (negative to –100 mV). The neurons fire spontaneously at 1–3 Hz, (c) and during DA application they develop an outward current (d) due to D_2 -mediated activation of GIRK channels

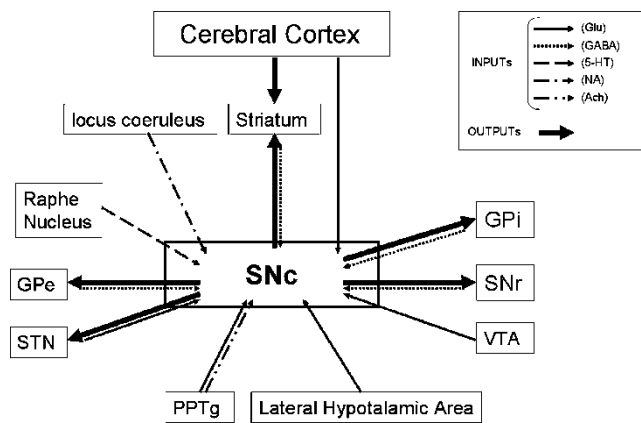


Fig. 3 Schematic model of the synaptic connections of SNc neurons. The neurotransmitters involved in the synaptic connections (*Glu* Glutamate, *GABA* γ -aminobutyric acid, *5-HT* Serotonin, *NA* Noradrenaline, *Ach* Acetylcholine) are indicated by different arrows (see insert). *Thick arrows* indicate outputs connections of SNc neurons to different brain regions; *thin arrows*, inputs to SNc neurons. Abbreviations: *GPe* External globus pallidus, *GPI* Internal globus pallidus, *STN* Subthalamic nucleus, *PPTg* Pedunculopontine tegmentum, *VTA* Ventral tegmental area, *SNr* Substantia nigra pars reticulata

pontine tegmentum (PPTg), and lateral preoptic- rostral hypothalamic area (Bezard and Gross 1998; Naito and Kita 1994; Reese et al. 1995; Smith et al. 1996) (Fig 3). Recently, a glutamatergic neuronal population has also been described within the adjacent ventral tegmental area (Yamaguchi et al. 2007) that may provide a local glutamatergic input to DAergic neurons. Some authors also propose that DAergic neurons of the VTA and SN may corelease glutamate, as these neurons were found to express the vesicular glutamate transporter VGLUT2 (Mendez et al. 2008). Interestingly, a large amount of data suggest an important role for NMDA receptor-mediated glutamatergic transmission in the regulation of burst firing (Johnson et al. 1992).

The DAergic cells also receive serotonergic (5-HT) projections from the medial and dorsal raphe nuclei (Hauber 1998; reviewed by Blandini et al. 2000) and noradrenergic (NA) inputs from the locus coeruleus. Besides glutamate, the PPTg provides a source of acetylcholine to the SN. Moreover, substance P is colocalized with GABA in the striato-nigral GABAergic terminals, and a local source of the peptides enkephalin and nociceptin affects cellular membrane properties and the release of DA. In addition, a peptidergic input from the lateral hypothalamus releases orexin on these cells. Another regulation of synaptic activities in the ventral mesencephalon derives from the endocannabinoid and endovanniloid systems (Marinelli et al. 2003; Marinelli et al. 2007). All these neurotransmitters control firing activity and consequently DA release locally and at distal sites.

It is believed that the firing activity of the DAergic cells results from the interaction between synaptic inputs and the intrinsic membrane properties, both contributing to modulate their firing rate, and therefore, increase or decrease DA release in the extracellular space (Grillner and Mercuri 2002). Whatever are the connecting elements of the basal ganglia, the regular/low-firing activity of SN DAergic cells and subsequent tonic DA release and DA receptor activation in the striatum regulate muscle tension and readiness of movements. By contrast, bursting activity, by producing a phasic stimulation of striatal DA receptors, facilitates rapid voluntary movements.

Electrophysiological Properties of Substantia Nigra Reticulata Neurons

Different from pars compacta DAergic neurons, SNr GABAergic neurons present a modest sag potential in response to hyperpolarizing current (Fig 4). The tonic firing pattern is dependent on several conductances, such as a slowly inactivating, voltage-dependent, tetrodotoxin (TTX)-sensitive Na^+ current and a TTX-insensitive inward current that is partly mediated by Na^+ . Moreover, an apamin-sensitive spike afterhyperpolarization, mediated by small-conductance Ca^{2+} -dependent K^+ (SK) channels, is also important for the precision of the autonomous neuronal activity (Atherton and Bevan 2005).

SNr neurons display a relatively depolarized membrane potential compared with other neuronal types, and this is very likely dependent on a tonic inward current (mediated by transient receptor potential channels, TRPC3) that provides a membrane depolarization of about 10 mV (Zhou et al. 2008). Unfortunately, the tonic and regular firing pattern of SNr cells controlled by inhibitory and excitatory inputs (Lestienne and Caillier 1986) can change in pathological conditions. Thus, in Parkinson's disease or after DAergic denervation, GABAergic reticulata as well as GPi neurons acquire a burst firing pattern. It has been proposed that a shift toward an irregular and burst firing pattern may be caused, at least in part, by an increased excitatory input from the STN (Albin et al. 1989; DeLong 1990; Bergman et al. 1994; Bergman et al. 1998; Shen and Johnson 2006). Indeed, NMDA receptor stimulation in these cells activates a calcium- activated nonselective cation conductance (TRPM2, Tepper and Lee 2007) that generates a plateau potential that may sustain the bursting behavior. The bursting activity of SNr neurons also depends on hyperpolarization-activated cation (HCN) current, Ca^{2+} -dependent small-conductance K^+ (SK), and high-voltage-activated Ca^{2+} (HVA) channels (Ibáñez-Sandoval et al. 2007).

Fig. 4 Electrophysiological properties of SNr GABAergic neurons recorded in in vitro rat slices. These neurons lack the I_h current (a) and the “sag” (b) at hyperpolarized membrane potentials. They fire tonically at ≈ 25 Hz and they are insensitive to DA and D2 agonists (quinpirole) (D)

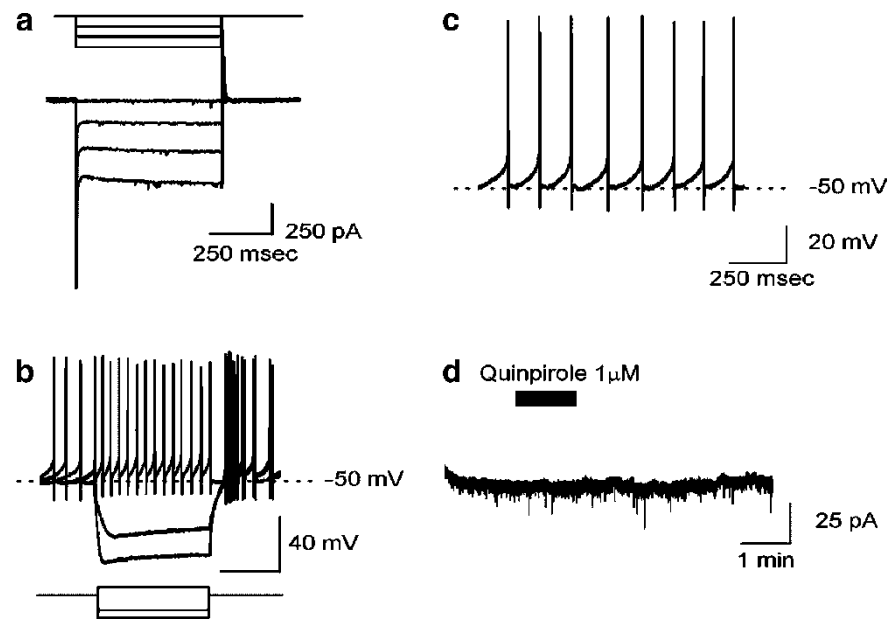
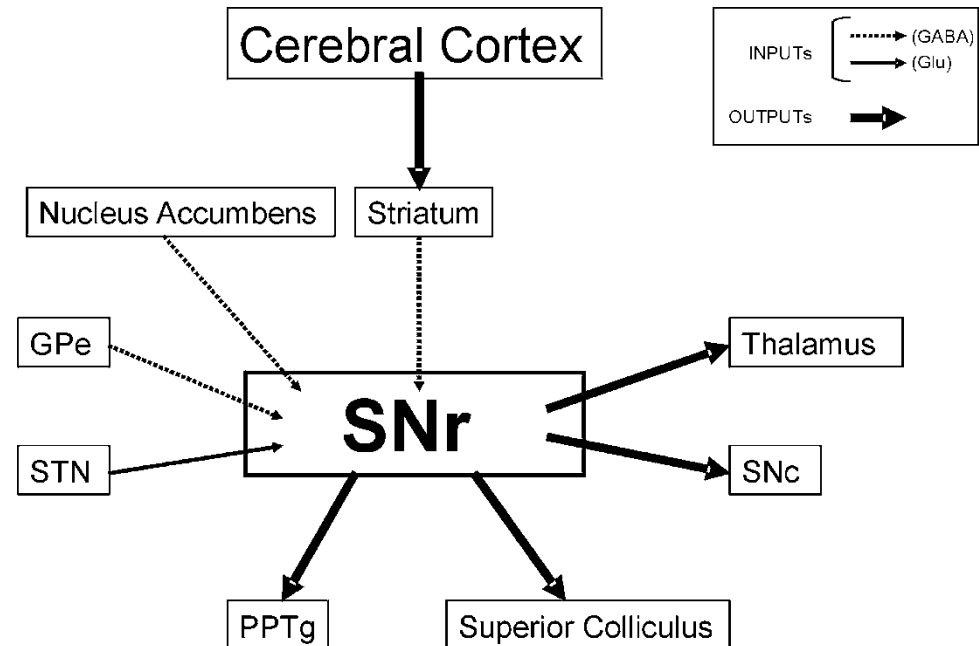


Fig. 5 Schematic model of the synaptic connections of SNr neurons. The neurotransmitters involved in the synaptic connection (*Glu* Glutamate, *GABA* γ -aminobutyric acid) are indicated by different arrows (see insert). *Thick arrows* indicate outputs connections of SNr neurons to different brain regions; *thin arrows*, inputs to SNr neurons. Abbreviations: *GPe* External globus pallidus, *STN* Subthalamic nucleus, *PPTg* Pedunculopontine tegmentum, *SNc* Substantia nigra pars compacta



Synaptic Connections of the Substantia Nigra Pars Reticulata

Together with the GPi, the SNr represents the output nucleus of the basal ganglia (Oertel and Mugnaini 1984). The SNr is mainly composed of GABAergic neurons, located ventral and adjacent to the SNc, and these provide a direct inhibition to thalamo-cortical cells and SNc DA neurons, the pedunculopontine nucleus, and the superior colliculus (SC) (Parent and Hazrati 1995) (Fig 5). Inhibitory SNr neurons normally

suppress inappropriate or unwanted behavior (Chevalier and Deniau 1990). A facilitation of movements would be expected to correlate with a reduction in the tonic firing rate of these cells. SNr GABAergic neurons are tonically active both in vivo (Wilson et al. 1977; Deniau et al. 1978; Guyenet and Aghajanian 1978) and in vitro (Nakanishi et al. 1987; Lacey et al. 1989) firing at ~ 25 Hz (Gernert et al. 2004). Within the motor thalamus, SNr cells provide a tonic inhibition onto glutamatergic thalamo-cortical neurons, which control voluntary movements.

The activity of SNr cells is regulated by inhibitory and excitatory inputs arising in a variety of brain areas. The main source of inhibitory projection is the striatum (Parent and Hazrati 1995), while the other sources include the GP (Smith and Bolam 1989) and nucleus accumbens (Deniau et al. 1994). Excitatory inputs to these cells mainly originate in the STN (Kita and Kitai 1987), which strongly regulates nigral activity.

The electrical stimulation of the SNr increases the GABA level and causes monosynaptic inhibitory potentials (IPSPs) in the thalamus (Timmerman and Westerink 1997; Ueki et al. 1977). Of note, this inhibitory nigro-thalamo-cortical pathway is also important in inhibiting the neuronal synchronization occurring during absence seizures (Paz et al. 2007).

Similarly, the electrical stimulation of the SNr evokes IPSPs in neurons of the PPTg (Saitoh et al. 2003). The GABAergic projection from the SNr to the PPTg is very likely involved in the control of rapid eye movements (REM) with atonia, signs that indicate REM sleep (Takakusaki et al. 2004).

The GABAergic projection from the SNr to neurons of the SC is involved in the regulation of saccadic eye movements that realign the center of gaze to objects of interest (Hikosaka et al. 2000; Sparks 1986). Voluntary saccades arise from eye fields of the prefrontal cortex (FEF) (Bruce and Goldberg 1985; Bruce et al. 1985; Sommer and Wurtz 1998; Sommer and Wurtz 2000). Caudate neurons containing GABA (Gerfen 1985) are activated by the FEF input and are directly connected to the SNr (Hikosaka and Sakamoto 1986; Hikosaka et al. 1989). Therefore, the discharge of caudate neurons results in a decrease in the activity of SNr neurons that disinhibit glutamatergic SC neurons. Recent evidence has also shown the existence of GABAergic interneurons within SC (Lee et al. 2007). In addition, it has been recently proposed that a direct connection between SNr neurons and these GABAergic interneurons provides a local inhibition to SC glutamatergic neurons projecting to the brainstem gaze center (Kaneda et al. 2008).

Dopamine Effects into the Dorsal Striatum

Within the basal ganglia, the functional balance between the direct and indirect pathways of striatal projections to output nuclei is controlled by DA, which is principally released by the terminals of SNc neurons. Dopamine acts on D₁ and D₂ receptors expressed by medium spiny striatal neurons. The DA receptors are mainly segregated, since D₁ is expressed in the striato-GPi / striato-nigral (direct pathway) and D₂ in the striato-GPe (indirect pathway) neurons. Both D₁ and D₂ receptors are G-protein coupled. The D₁ family (including D₁ and D₅ receptors), coupled to G_{αs}, stimulates adenylate cyclase, while the D₂ family (including D₂, D₃ and D₄

receptors), coupled to G_{αi/o}, inhibits it (reviewed by Bernardi and Mercuri 2009). At cellular level, the activation of D₁ receptors leads to increased levels of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA) (Flores-Hernandez et al. 2000). On the other hand, the D₂-dependent effects include decreased levels of cAMP and the inhibition of PKA and an eventual activation of phospholipase C-inositol triphosphate (IP₃) with changes in intracellular calcium levels.

The dichotomy in the cAMP formation might result in opposite consequences on neuronal excitability (Bernardi and Mercuri 2009). Early electrophysiological data have suggested a predominant inhibitory effect of DA on low-firing medium spiny neurons, most likely mediated by D₁ receptors. This inhibitory effect is due to the reduction of voltage-dependent inward currents (sodium-mediated) and to the modulation of cortico-striatal synaptic transmission via c-AMP-dependent PKA activation. On the contrary, the stimulation of D₂ receptors inhibits both glutamate- and GABA-induced currents and voltage-dependent calcium currents (Hernandez-Lopez et al. 2000).

It is generally accepted that the release of DA in the striatum either stimulates movement along the direct pathway, by acting on D₁-expressing spiny neurons, or inhibits movement by acting on the indirect pathway that includes D₂-expressing spiny neurons (DeLong and Wichmann 2007). However, this is still controversial, because the currently used antiparkinsonian drugs are D₂ agonists.

According to the theory, the effects caused by DA on striatal neurons that make up the direct pathway result in a net reduction in GPi and SNr neuronal activity and finally in a tonic disinhibition of the glutamatergic thalamo-cortical neurons. By contrast, a DA-mediated activation of the indirect pathway, by disinhibiting subthalamic cells, increases the activity of the output basal ganglia centers (GPi / SNr) causing, via thalamic cells, the inhibition of movement. This interpretation relies on a model of basal ganglia circuitry proposed by many authors (Albin et al. 1989; Alexander and Crutcher 1990; Graybiel 1990) who have considered the direct and indirect pathways, connecting the striatum to the output nuclei, completely separate. Experimental evidence has subsequently suggested that this segregation is not entirely true, since striatal spiny neurons possess different local collaterals that synaptically connect the two spiny neuronal populations (Yung et al. 1996). Moreover, the GPe not only projects to the STN but also sends terminals to GPi and SNr (reviewed by Smith et al. 1998) without interposition of the STN. In addition, STN directly receives cortical inputs.

Rendering the picture describing the effects of DA in the striatum even more complex, this catecholamine regulates long-term synaptic plasticity in the cortico-striatal excitatory pathway. In fact, both long-term depression (LTD) (Calabresi

et al. 1992; Calabresi et al. 2000; Choi and Lovinger 1997a, b) and long-term potentiation (LTP) (Akopian et al. 2000; Calabresi et al. 1996) of the excitatory postsynaptic potentials (EPSP) have been described and are modulated by DA. Indeed, corticostriatal LTD and LTP, among other factors (Choi and Lovinger 1997a; Gerdeman et al. 2002; Ronesi et al. 2004), require DA receptor stimulation (mainly D₂ for LTD and D₁ for LTP) (Wang et al. 2006; Calabresi et al. 1992; Calabresi et al. 1997).

Moreover, a D₂-mediated inhibition of cholinergic tonically active interneurons (TANs) regulates LTD by lowering acetylcholine release in the striatum and facilitating, via muscarinic M₁ receptors, endocannabinoid production and presynaptic inhibition, via CB₁ receptor activation (Wang et al. 2006).

Dopamine Effects into the STN, GP and SNr

Another important challenge for the “classical” model of basal ganglia circuitry came with the discovery that a physical connection exists between the SNc, STN (Hassani et al. 1996; Cossette et al. 1999), and GP (Lindvall and Bjorklund 1979; Cossette et al. 1999; Parent et al. 2000). Particularly, it has been shown that two types of nigrostriatal DAergic fibers exist (Gauthier et al. 1999): type 1 fibers travel directly to the striatum without emitting collaterals along their way and branch abundantly within a limited sector of the striatum; type 2 arborize extensively within various extra-striatal structures, including the two segments of the GP and STN, before reaching the striatum with poorly branched collaterals. These two axonal systems allow single SNc neurons to have different effects on striatal neurons and to act directly upon one or more of the extrastriatal components of the basal ganglia (Parent et al. 2000). Based on the leading anatomical considerations, it has been suggested that DA receptor agonists could alter STN neuronal activity indirectly, predominantly via D₂ receptors expressed in the striatopallidal pathway (Gerfen et al. 1990). However, DA directly alters neuronal firing in the STN (Blandini et al. 2000; Baufreton et al. 2005). Indeed, by activating DA receptors, the catecholamine strengthens regular, single spike firing. In fact, the activation of STN D₂ receptor family directly depolarizes and excites neurons (Zhu et al. 2002; Ramanathan et al. 2008) but prevents spontaneous burst-firing. Moreover, it has been recently suggested that DA acting at presynaptic D₂-like receptors reduces the propensity for GABAergic transmission to generate correlated, bursting activity in STN neurons (Baufreton and Bevan 2008).

On the other hand, it has been shown that the STN excitation mediated by the D₁/D₂ agonist apomorphine requires the activation of D₁ receptors, since it was pre-

vented by selective D₁ receptor antagonist; interestingly, a D₂-mediated tone is also required, because D₁ firing activation in the presence of D₂ antagonist was completely abolished (Kreiss et al. 1996; Kreiss et al. 1997). Others have reported that D₅ receptors facilitate burst firing in a subset of subthalamic neurons (Baufreton et al. 2003).

SNc neurons release DA also directly in the SNr from their dendrites that descend dorso-ventrally and arborize profusely along the basis of the SNr (Arsenault et al. 1988; Bjorklund and Lindvall 1975). Once released within SNr, DA interacts with D₁ receptors (Richfield et al. 1987). Most of these receptors are located on pre-synaptic axons and axon terminals of GABAergic striatonigral projections. It has been recently shown that the stimulation of D₁ receptors significantly reduced discharge rates in SNr and also in GPi neurons, whereas injections of the D₁ antagonist SCH23390 increased firing in the majority of GPi neurons. Microdialysis measurements of GABA concentrations in GPi and SNr showed that the DA agonist increased the level of this transmitter (Kliem et al. 2007).

Both the findings are compatible with the hypothesis that D₁ receptors activation leads to GABA release from striatopallidal or striato-nigral afferents, which may secondarily reduce firing of basal ganglia output neurons (Kliem et al. 2007).

Pathological Consequences of Dopamine Denervation in Parkinson's Disease

The progressive degeneration of DAergic neurons in the SNc and the consequent drop of the level of DA in brain areas innervated by DAergic fibers characterize Parkinson's disease (PD). Within the basal ganglia, DA depletion mainly occurs in the striatum, the STN, the GP, and the SN. Animal models of PD, obtained by the intracerebral administration of 6-hydroxydopamine (6-OHDA) (in rodents) or by the systemic injection of the neurotoxin 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) in nonhuman primates show permanent loss of mesencephalic DA neurons, which result in a PD-like syndrome (DeLong 1990). These models represent a good substrate to perform functional studies after DA depletion in the basal ganglia circuitry. An excessive inhibition of basal ganglia targets (thalamic neurons), via the SNr and GPi, has been suggested to underlie symptoms of PD (Albin et al. 1989; DeLong 1990).

The most prominent alteration described in PD models and also in humans is increased firing activity (Bergman et al. 1994; Kreiss et al. 1997), glucose metabolism (Mitchell et al. 1989), and mitochondrial enzyme activity (Vila et al. 1996; Vila et al. 1999) in STN glutamatergic neurons. Others have reported that a markedly dysregulated state of striatal

activity develops after chronic DA denervation and in such a pathological state of medium spiny neurons activity, DA induces altered and disproportionate responses.

It has been found that procedures aimed at limiting subthalamic neuronal output possibly by reducing SNr and GPi activity reverse the behavioral effects of DA depletion in rats (Anderson et al. 1992; Blandini et al. 1995; Delfs et al. 1995), primates (Bergman et al. 1990; Aziz et al. 1991; Benazzouz et al. 1993), and humans (Benabid et al. 1994).

What are the causes of increased STN activity in DA-depleted conditions? One possibility is that the loss of DA in the striatum leads to a reduction in the activity of the inhibitory GABAergic external pallido-subthalamic pathway (Miller and DeLong 1988; Albin et al. 1989). However, in contrast to this hypothesis, experimental evidence has shown that, under conditions of chronic DA depletion, some aspects of pallidal neuronal activity seem to be augmented, neuronal bursting activity is increased (Pan and Walters 1988), and levels of mRNA for the GABAergic metabolic enzyme GAD67 are elevated (Kincaid et al. 1992; Soghomonian and Chesselet 1992; Delfs et al. 1995). Another possibility to explain the increased neuronal activity in the STN is the loss of DAergic tone at DA receptors located in the subthalamus (Delfs et al. 1995). However, this hypothesis implies that DA receptors in the STN exert an inhibitory effect on neuronal activity, which is in contrast with the evidence that DA excites STN neurons (Kreiss et al. 1996).

It has to be considered that the STN-increased activity described in PD may also have a negative effect onto the DAergic neurons in the SNc. Indeed, the activation of the glutamatergic pathway that connects the STN to the SNc (Smith et al. 1996) would provide an overexcitation of residual DAergic neurons in the pars compacta, thus resulting in further nigral excitotoxic damage. Thus, alterations in the STN neuronal activity would affect both PD motor symptoms and the progression of nigral degeneration.

Based on the “rate model” that considers Parkinsonism as a condition of altered neuronal firing rate in the basal ganglia (DeLong and Wichmann 2007), these inconsistencies are difficult to explain. Another consideration is that altered firing patterns may be more important than changes in firing rate. In fact, two important modifications occurring in the basal ganglia of PD patients are the development of oscillatory phenomena and the appearance of synchrony among neurons (Bergman et al. 1998). Indeed, oscillatory activities at 15–30 Hz (beta-range) are present in the STN, GPi, and SNr in animal PD models and PD patients being suppressed by therapies that reestablish the DAergic tone (Brown et al. 2001; Williams et al. 2002).

It has been recently shown that the oscillatory phenomena appear only after chronic DA depletion (Mallet et al. 2008) and not during the acute suppression of DA functions. This

suggests that there are plastic consequences induced by a long-term and progressive DA depletion.

Since DA is a key element in determining the plasticity of the cortico-striatal excitatory pathway, it is believed that aberrant forms of synaptic plasticity underlay the clinical aspects of hypodopaminergia.

It has been reported that in animal models of DA denervation, striatal LTD is lacking and replaced by LTP (Calabresi et al. 1992; Kerr and Wickens 2001). Interestingly, the D₂ knockout animals, which present altered synaptic plasticity, have some clinical features of PD (Baik et al. 1995; Calabresi et al. 1997). The data on synaptic plasticity confirm the role of DA in the regulation of neuronal activity in the striatum, suggesting that when a deficit of DA is present, the alteration of the LTD/LTP sequence might represent the cellular substrate for most of the hypokinetic symptoms observed in PD.

Moreover, it has been reported that LTP of cortico-striatal synapses is diminished by a low-frequency stimulation protocol that causes depotentiation in control animal or DA-denervated animals chronically treated with L-DOPA. This striatal depotentiation was dependent on protein phosphatase activation and inhibited by D₁ agonists through adenylate cyclase activation (Picconi et al. 2003). However, in a rat model of dyskinesia, (DA-denervated animals chronically treated with levodopa but showing hyperkinetic movements), LTP was resistant to depotentiation.

Conclusions

This short excursus on SN functions, with respect to the integrative actions occurring in the basal ganglia, indicates that both SNc and SNr cells are important stations for the physiological control of movement. Consequently, pathological states occur when there is an alteration in their functioning. It is clear that a loss of SN DAergic neurons causes several changes in the basal ganglia circuitry. Particularly, the output nuclei (GPi and SNr) become hyperactive and oscillate; this may possibly cause an over-inhibition of the thalamo-cortical neurons. This neuronal hyperactivity is partially sustained by an enhanced glutamatergic input from the STN to the GPi and the SNr, caused by a diminished DAergic tone that could directly or indirectly affect STN activity. Based on these considerations, the therapeutic strategies aimed at antagonizing basal ganglia dysfunctions in PD might involve antidegenerative, reparative, substitutive/pharmacological approaches that correct the deficit of the nigro-striatal DAergic pathway. An additional strategy is a functional/pharmacological approach to normalizing activity directly in the GPi/SNr complex.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 8

Electrophysiological Characteristics of Dopamine Neurons: A 35-Year Update

Wei-Xing Shi

Abstract This chapter consists of four sections. The first section provides a general description of the electrophysiological characteristics of dopamine (DA) neurons in both the substantia nigra and ventral tegmental area. Emphasis is placed on the differences between DA and neighboring non-DA neurons. The second section discusses the ionic mechanisms underlying the generation of action potential in DA cells. Evidence is provided to suggest that these mechanisms differ not only between DA and non-DA neurons but also between DA cells located in different areas, with different projection sites and at different developmental stages. Some of the differences may play a critical role in the vulnerability of a DA neuron to cell death. The third section describes the firing patterns of DA cells. Data are presented to show that the current “80/160 ms” criteria for burst identification need to be revised and that the burst firing, originally described by Bunney et al., can be described as slow oscillations in firing rate. In the ventral tegmental area, the slow oscillations are, at least partially, derived from the prefrontal cortex and part of prefrontal information is transferred to DA cells indirectly through inhibitory neurons. The final section focuses on the feedback regulation of DA cells. New evidence suggests that DA autoreceptors are coupled to multiple effectors, and both D1 and D2-like receptors are involved in long-loop feedback control of DA neurons. Because of the presence of multiple feedback and nonfeedback pathways, the effect of a drug on a DA neuron can be far more complex than an inhibition or excitation. A better understanding of the intrinsic properties of DA neurons and their regulation by afferent input will, in time, help to point to the way to more effective and safer treatments

for disorders including schizophrenia, drug addiction, and Parkinson’s disease.

Keywords Amphetamine • Antipsychotic drug • A-type K^+ channel • Autoreceptor • Burst • Feedback control • I_H channel • Non-DA neurons • Pacemaker potential • Prefrontal cortex • SK channel • Slow oscillation • Striatum • Substantia nigra • Ventral tegmental area

Abbreviations

1-EBIO	1-ethyl-2-benzimidazolinone
l-dopa	3,4-dihydroxy-L-phenylalanine
4-AP	4-aminopyridine
6-OHDA	6-Hydroxydopamine
AHP	Afterhyperpolarization
BLA	Basolateral amygdale
CB	Calbindin
cAMP	Cyclic adenosine monophosphate
DA	Dopamine
ERG	Ether-a-go-go
GABA	Gamma-aminobutyric acid
HVA	High voltage-activated
IP3	Inositol triphosphate
IK	Intermediate conductance calcium-activated K channels
BK	Large conductance calcium-activated K channels
LVA	Low voltage-activated
mGluR	Metabotropic glutamate receptors
NAc	Nucleus accumbens
NMDA	N-methyl-D-aspartic acid
PFC	Prefrontal cortex
PKA	Protein kinase A
SO	slow oscillations
SK	Small conductance calcium-activated K channels
SN	Substantia nigra
VTA	Ventral tegmental area
TTX	Tetrodotoxin
TRP	Transient receptor potential
TH	Tyrosine hydroxylase

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Electrophysiological Identification of Dopamine Neurons

In Vivo Studies

Using *in vivo* single unit recording, Bunney, Aghajanian, and their colleagues provided the first evidence that dopamine (DA) neurons in the substantia nigra (SN) and the ventral tegmental area (VTA) can be distinguished from neighboring non-DA neurons based on their electrophysiological and pharmacological properties (Aghajanian and Bunney 1973; Bunney et al. 1973a; Bunney et al. 1973b; Guyenet and Aghajanian 1978). They found that DA, but not non-DA, neurons are inhibited by systemic injection of direct or indirect DA agonists such as apomorphine, d-amphetamine, and l-dopa. The inhibition is reversed by DA antagonists, including chlorpromazine and haloperidol, suggesting a DA receptor-mediated effect. Compared with non-DA neurons, DA neurons have a broader action potential (Fig. 1), slower conduction velocity, and lower firing rate. In chloral hydrate anesthetized animals, some DA neurons also exhibit a firing pattern not observed in neighboring non-DA neurons, which Bunney and colleagues called burst firing (Fig. 1). These differences between DA and non-DA neurons have since been confirmed by numerous studies and widely used as the criteria to identify DA

and non-DA neurons in the SN and VTA. Intracellular labeling combined with fluorescence histochemistry confirms that DA neurons, identified based on these criteria, contain DA (Grace and Bunney 1983) and have the ability to convert exogenously applied l-dopa into DA (Grace and Bunney 1980).

However, evidence suggests that some DA neurons in the VTA do not fully meet the criteria mentioned earlier. Chiodo et al. (1984) showed that a small percent of VTA DA cells is insensitive to the inhibitory effect of low doses of apomorphine, suggesting a lack of DA autoreceptors on these cells. Evidence further suggests that DA cells lacking the inhibitory DA autoreceptor project to the prefrontal cortex (PFC) or cingulate cortex (see also Lammel et al. 2008). Other investigators suggest, however, that at least, some DA neurons projecting to the PFC express the inhibitory DA autoreceptor (Shepard and German 1984; Gariano et al. 1989a; 1989b). Using extracellular recording combined with juxtacellular labeling, Ungless et al reported that a small group of VTA DA neurons exhibits narrow action potentials (Ungless et al. 2004). Similar results were reported by Luo et al. (2008). The latter authors also identified a novel group of fast-firing VTA non-DA cells (>10 spikes), which exhibits wide action potentials, is inhibited by the D2 agonist quinpirole, but is immuno-negative for tyrosine hydroxylase (TH) and glutamic acid decarboxylase.

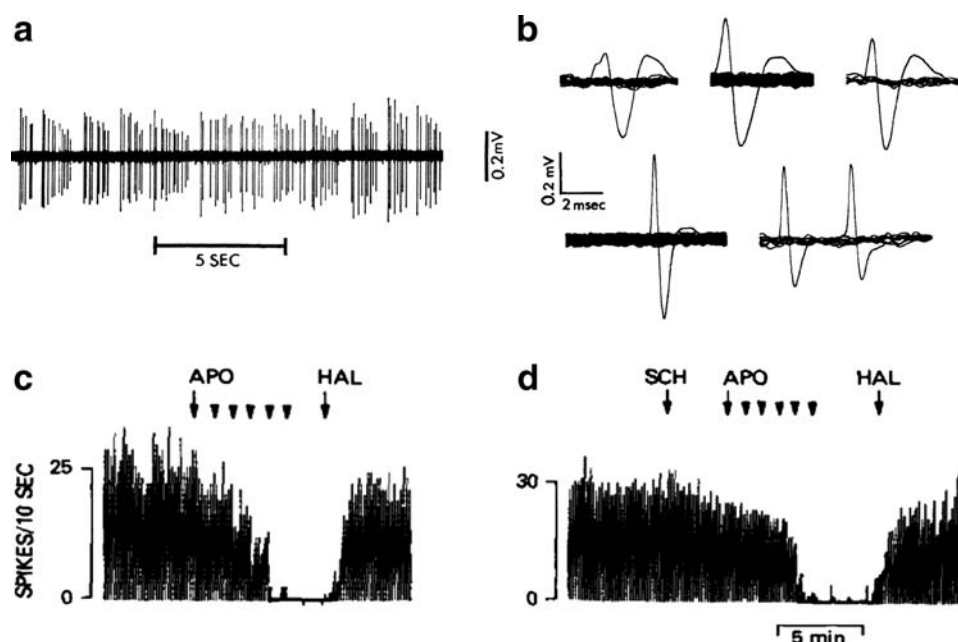


Fig. 1 Characteristics of DA neurons recorded *in vivo*. (a) The first published recording of a rat DA neuron (from Bunney et al. 1973b). The slow firing rate and rhythmic burst-like activity are characteristics of DA cells recorded in a chloral hydrate-anesthetized rat. (b) Typical action potentials, recorded extracellularly, from DA (top three) and non-DA neurons (bottom two) in the SN (from Guyenet and Aghajanian 1978). (c) Rate histogram showing inhibition of a DA neuron induced by systemic injection of low doses of apomorphine (cumulative dose: $40\mu\text{g kg}^{-1}$) and the reversal of the inhibition by haloperidol ($50\mu\text{g kg}^{-1}$). (d) Rate histogram showing that apomorphine ($40\mu\text{g kg}^{-1}$), given after the D1 antagonist SCH23390 (1 mg kg^{-1}), can still inhibit a DA cell and the inhibition is reversed by haloperidol (from Carlson et al. 1986).

It is important to point out that when recorded extracellularly, the shape of an action potential can vary significantly depending on the impedance, size, and position of the recording electrode relative to a recorded cell. The shape of an action potential can also be affected by the signal-to-noise ratio and the filter settings of an amplifier. Thus, criteria developed by one lab concerning action potential shape may not be directly applicable to recordings from a different lab. It is also worth pointing out that the firing pattern of DA neurons can differ between different preparations. The burst firing, originally described by Bunney et al. (1973b), is more frequently observed in chloral hydrate-anesthetized rats than in locally anesthetized, paralyzed rats.

In Vitro Studies

SN DA neurons recorded in vitro exhibit properties similar to those observed in vivo, including a broad action potential, slow firing rate, and inhibitory response to DA agonists (Pinnock 1984; Sanghera et al. 1984). They, however, fire in a highly regular pattern and show no bursting when recorded in brain slices (Sanghera et al. 1984). The regularity of firing is further enhanced after synaptic transmissions are decreased by a perfusion medium containing high Mg^{2+} and low Ca^{2+} . These results suggest that irregular firing observed in vivo, including bursting, is caused by synaptic input to DA neurons.

Intracellular recordings in brain slices confirm that the action potential in DA neurons is much broader than those recorded from adjacent non-DA cells (Kita et al. 1986; Nakanishi et al. 1987). The exact reason is still unclear, but it may be related to the fact that the axon in DA neurons usually emerges from a dendrite and not from the soma and the distance between the soma and the dendritic site from which the axon emerges can be as long as 240 μm (Hausser et al. 1995). Simultaneous somatic and dendritic recordings show that the action potential always starts in the axon and then spreads through the axon-bearing dendrite to the soma. The long distance between the site of spike initiation and the soma may partially account for the slow rising phase of the action potential recorded from DA cell soma (Hausser et al. 1995). In non-DA neurons, the axon usually emerges from a site near the soma and the action potential occurs first in the soma and then dendrites (Hausser et al. 1995; Atherton and Bevan 2005).

Intracellular recordings have also revealed several other properties of DA cells that are not observed or less pronounced in non-DA neurons in the same areas. These properties include a Ca^{2+} -dependent pacemaker potential (Fig. 2), a slowly developing inward rectification (sag) in response to hyperpolarization (Fig. 3), and an outward recti-

fication (Fig. 3) evoked by depolarization steps from a hyperpolarized level (Pinnock 1985; Kita et al. 1986; Nakanishi et al. 1987). As will be discussed, the inward rectification, also known as the anomalous rectifier, is mediated through the hyperpolarization-activated, cyclic nucleotide-gated cation (HCN or I_H) channel. The outward rectification, also known as the transient outward rectifier, is due to the activation of A-type K^+ (I_A) channels. Studies in brain slices further show that DA hyperpolarizes DA neurons by increasing G protein-coupled K^+ conductance, and the effect is observed in DA and not in non-DA neurons (Lacey et al. 1987, 1989). Immunostaining confirms that cells displaying the properties mentioned earlier are DAergic (Grace and Onn 1989; Yung et al. 1991; Johnson and North 1992; Richards et al. 1997). However, as will be discussed, some degree of heterogeneity has been observed among DA neurons regarding those properties.

Ionic Mechanisms Underlying Spike Generation in DA Neurons

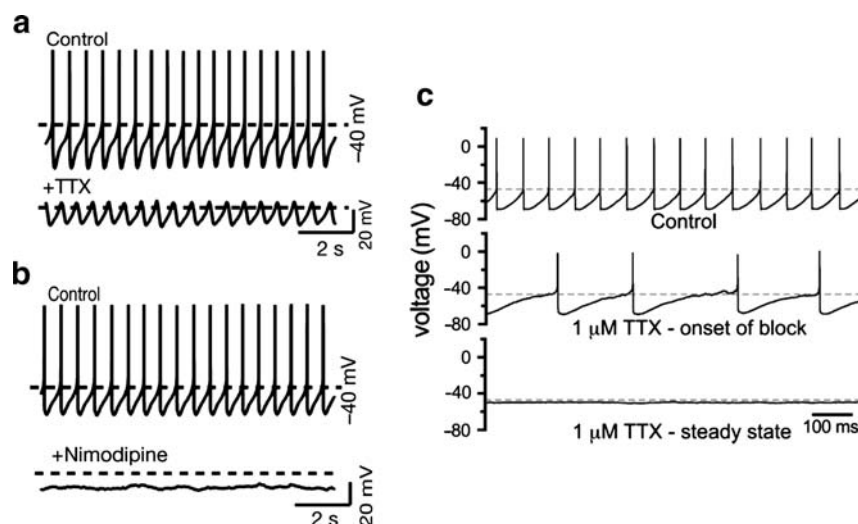
Pacemaker Potential

Some DA neurons are capable of firing action potentials spontaneously even after synaptic inputs are completely blocked, suggesting the presence of pacemaker mechanisms in these cells. Several ion channels contribute to pacemaking in DA neurons, including Na^+ , Ca^{2+} , K^+ , and nonselective cation channels. The role of these channels varies depending on the age of the animal, differs between VTA and SN DA neurons, and can be altered by drug treatment.

Voltage-sensitive Na^+ Channels

The contribution of Na^+ channels to pacemaking in DA neurons was first suggested by the finding that TTX not only blocks the fast action potential but also decreases the slow depolarization preceding the action potential (Grace and Onn 1989; Kang and Kitai 1993b). The Na^+ channel blocker 202W92 also slows the spontaneous firing rate of DA cells (Caputi et al. 2003). However, a major portion of the pacemaker potential, expressed as a slow oscillatory potential, persists in the presence of TTX or a high concentration of 202W92 (Fig. 2a, Fujimura and Matsuda 1989; Harris et al. 1989; Yung et al. 1991; Nedergaard et al. 1993; Chan et al. 2007). Puopolo et al. (2007) show that both Ca^{2+} and Na^+ currents contribute to the spontaneous interspike depolarization in SN DA neurons, with Ca^{2+} current carrying about twice as much charge as Na^+ current. Chan et al.

Fig. 2 The pacemaker potential in DA and non-DA neurons. (a) Whole cell recordings from an adult mouse SN DA neuron showing that TTX inhibits the fast action potential and leaves the pacemaker potential largely intact. (b) The L-type Ca^{2+} channel blocker nimodipine inhibits the spontaneous firing and abolishes the underlying pacemaker potential (modified after Chan et al. 2007). (c) Recordings from a SN non-DA neurons showing that TTX inhibits both the fast action potential and the underlying pacemaker potential (from Atherton and Bevan 2005).



(2007) further suggest that Na^+ channels are essential for pacemaking in VTA DA neurons and juvenile SN DA neurons, but they are not required in adult SN DA neurons.

Some non-DA neurons in the SN are also spontaneously active when synaptic inputs are pharmacologically blocked (Atherton and Bevan 2005). Unlike SN DA cells, non-DA neurons show no oscillations in the membrane potential in the presence of TTX (Fig. 2c, Yung et al. 1991; Atherton and Bevan 2005). In some DA neurons, TTX also inhibits the spontaneous pacemaker potential. In those DA cells, however, depolarizing currents or the release from hyperpolarizing currents can usually induce membrane oscillations (Nedergaard and Greenfield 1992; Nedergaard et al. 1993). In non-DA neurons, the same manipulations produce no effect, indicating that the TTX-sensitive Na^+ channel is absolutely required for pacemaking in SN non-DA neurons (Atherton and Bevan 2005).

Voltage-sensitive Ca^{2+} Channels

Voltage-gated Ca^{2+} channels are formed as a complex of several different subunits: $\alpha 1$, $\alpha 2\delta$, $\beta 1-4$, and γ . The $\alpha 1$ subunit forms the Ca^{2+} selective pore and is the principal determinant of gating and pharmacology. A total of ten $\alpha 1$ subunits have been identified. They can be divided into three main groups: Ca_v1 (L-type), Ca_v2 (P/Q-type, N-type, and R-type), and Ca_v3 (T-type). DA neurons appear to express all but the R-type of Ca^{2+} channels (Takada et al. 2001). However, local perfusion of a R-type channel blocker has been shown to decrease DA release in the SN (Bergquist and Nissbrandt 2003; but see Chen et al. 2006). Available evidence suggests that both the L- and P/Q-types of channels contribute to the depolarizing phase of the pacemaker poten-

tial, whereas the T-type of channels may be involved in both the depolarizing and repolarizing phases of the potential.

The L-type of Ca^{2+} channels are selectively blocked by dihydropyridines including nifedipine. Early studies suggest that nifedipine has no effect or only a small effect on SN DA neurons (Fujimura and Matsuda 1989; Kang and Kitai 1993a). Subsequent studies show that dihydropyridines inhibit both the spontaneous firing and the pacemaker potential persistent in the presence of TTX (Fig. 2b, Nedergaard et al. 1993; Mercuri et al. 1994; Chan et al. 2007; Puopolo et al. 2007). Chan et al suggest that Na^{2+} channels play a more critical role than Ca^{2+} channels in pacemaking in VTA DA neurons, since TTX blocks the pacemaker potential in those cells (Chan et al. 2007). Other studies suggest, however, that the L-type channels are also required for pacemaking in VTA DA neurons (Ugedo et al. 1988; Mercuri et al. 1994).

Traditionally, L-type channels are classified as high voltage-activated (HVA) channels, since they open only at relatively more depolarized potentials. This property makes them unsuited for pacemaking. In DA neurons, however, L-type channels contain mainly the $\text{Ca}_v1.3$ subunit (Takada et al. 2001; Chan et al. 2007). Different from the more widely distributed $\text{Ca}_v1.2$ -containing channels, the $\text{Ca}_v1.3$ -containing channels open at subthreshold membrane potentials. The finding that the inhibitory effect of dihydropyridines on SN DA neurons is abolished in $\text{Ca}_v1.3$ -knockout mice suggests that the effect, observed in wild-type mice, is mediated through the $\text{Ca}_v1.3$ -containing channels (Chan et al. 2007). Interestingly, in $\text{Ca}_v1.3$ -knockout mice, SN DA neurons continue to fire action potentials spontaneously. Although the activity is insensitive to dihydropyridines, it is blocked by TTX or the I_H channel blocker ZD 7288, suggesting a switch from a Ca^{2+} channel-based mechanism to a TTX- and ZD

7288-sensitive pacemaking. A similar change is observed in DA neurons from wild-type mice following a prolonged blockade of L-type channels by a dihydropyridine antagonist. Since the change is accompanied with a significant reduction in the sensitivity of the cell to 6-OHDA and MPTP, two toxins widely used to create experimental Parkinson's disease, it is suggested that L-type channel blockers may offer a neuroprotective effect in Parkinson's disease (Chan et al. 2007).

P/Q-type channels contain the $\alpha 1$ subunit CaV2.1 and are selectively blocked by the spider toxin ω -agatoxin IVA. In DA neurons, a major portion of the Ca^{2+} current evoked by a large depolarizing voltage pulse is blocked by ω -agatoxin IVA, suggesting that P/Q type channels are present in these cells (Cardozo and Bean 1995; Durante et al. 2004). Unexpectedly, ω -agatoxin IVA also slows the firing rate of SN DA neurons (Puopolo et al. 2007). How the blockade of this type of HVA Ca^{2+} channels leads to a decrease in the pacemaking activity in DA neurons remains unknown.

N-type channels contain the $\alpha 1$ subunit CaV2.2 and are selectively blocked by ω -conotoxins, including GVIA, MVIIA, and CVID. Although N-type channels are present in DA neurons (Kang and Kitai 1993a; Nedergaard et al. 1993; Cardozo and Bean 1995), the blockade of these channels produces no significant effect on pacemaking in DA neurons. This is consistent with the fact that N-type channels are of HVA type, are inactivated near the typical resting membrane potentials, and open only when the cell is depolarized to above the threshold for spiking (Puopolo et al. 2007).

T-type channels are low voltage-activated (LVA) Ca^{2+} channels containing either $\text{Ca}_v3.1$, 3.2, or 3.3. Since they are activated near typical resting membrane potentials, T-type channels are key contributors to the excitability of several types of spontaneously active neurons. They are blocked by mibefradil or low concentrations of Ni^{2+} and have a low sensitivity to Cd^{2+} . T-type channels are present on DA neurons (Kang and Kitai 1993a; Wolfart and Roeper 2002; but see Cardozo and Bean 1995). The blockade of these channels by low concentrations of Ni^{2+} was first reported to have no significant effect on DA neurons (Nedergaard et al. 1993). Wolfart and Roeper found, however, that the blockade not only decreases the firing rate but also slows the repolarization phase of the pacemaker potential. The latter effect further leads to an increased variability in firing, an effect similar to that produced by the Ca^{2+} -dependent K^+ channel blocker apamin. In a small subset of DA neurons, the blockade of T-type channels by Ni^{2+} converts single-spike firing into bursting of the cell (see Fig. 4b, Wolfart and Roeper 2002). As will be discussed, T-type channels in DA cells are functionally coupled to a subfamily of Ca^{2+} -activated K^+ channels called SK channels. Thus, the blockade of T-type channels also inhibits the functional expression of SK channels.

Other Channels

A number of other channels have also been suggested to play a role in pacemaking in DA neurons, including I_H , I_A , SK, and transient receptor potential (TRP) channels. I_H channels are inwardly rectifying, nonselective cation channels. There is evidence that I_H channels are critical to pacemaking in VTA DA neurons and juvenile SN DA neurons, but less important in adult SN DA neurons (Chan et al. 2007).

I_A channels are voltage-sensitive K^+ channels activated when the cell is depolarized from subthreshold voltages. The activation of these channels slows the rate of depolarization, leading to a delay in spiking. Thus, I_A channels play an inhibitory role in pacemaking in DA neurons. The blockade of these channels increases the firing of DA neurons (e.g., Nedergaard 1999; Liss et al. 2001).

Ca^{2+} influx during pacemaking not only depolarizes the cell but also activates Ca^{2+} -dependent K^+ channels. The latter is at least partially responsible for the repolarizing phase of the pacemaker potential. Consistent with this suggestion, apamin, a Ca^{2+} -dependent K^+ channel blocker, prolongs the depolarization so that each pacemaking cycle, which triggers only one action potential under control conditions, can now trigger multiple spikes in the presence of apamin (Ping and Shepard 1996; Wilson 2000, p. 353).

TRP channels are a family of loosely related ion channels that are nonselectively permeable to cations, including Ca^{2+} . In mammals, the TRP superfamily contains six subfamilies: classical (TRPC), vanilloid (TRPV), melastatin (TRPM), ANKTM1 (TRPA), muclopins (TRPML), and polycystins (TRPP). 2-aminoethoxy-diphenyl borate (2-APB) is a non-selective blocker of TRP channels, whereas SKF 96365 blocks mainly TRPC channels at low concentrations. Both drugs have been shown to inhibit pacemaking in SN DA neurons (Kim et al. 2007).

The Anomalous Rectification and I_H Channels

In response to large hyperpolarizing current pulses, DA neurons typically show a slowly developing, voltage-dependent inward rectification (Fig. 3a). This inward rectification, also known as the anomalous inward rectification, is mediated by the hyperpolarization-activated, cyclic nucleotide gated cation current I_H . Four I_H channel candidate genes have been identified: HCN1-4. SN DA neurons express mRNA for HCN2, HCN3, and HCN4 (Franz et al. 2000).

The anomalous inward rectification in DA neurons was first observed in vivo (Grace and Bunney 1983) and is more pronounced in neurons recorded in brain slices (Pinnock 1985; Kita et al. 1986; Grace and Onn 1989). I_H channels are predominantly expressed on dendrites (Franz et al. 2000). The reduced membrane conductance secondary to the

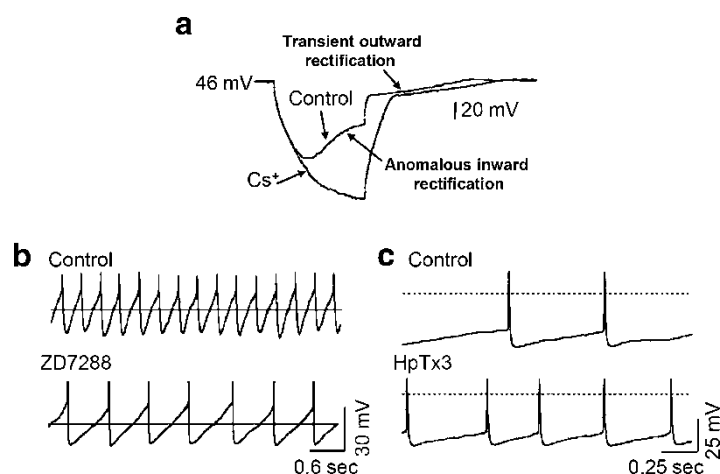


Fig. 3 The anomalous inward rectification and the transient outward rectification. (a) Intracellular recordings from a SN DA neuron showing both the anomalous inward rectification and the transient outward rectification induced by a hyperpolarization current step. Cs^+ blocks the anomalous inward rectification and has minimal effect on the outward rectification (modified after Harris 1992). (b) Recordings from a different SN DA neuron showing that the I_h channel inhibitor ZD7288 slows the spontaneous firing rate of the cell (modified after Neuhoff et al. 2002). (c) Recordings from another DA neuron showing that heteropodatoxin3 (HpTx3), a selective I_A channel blocker, increases the spontaneous firing rate of the cell (modified after Liss et al. 2001).

reduced synaptic activity may make I_H more readily detectable in brain slices. I_H has been shown to decrease when the membrane conductance is increased by the activation of either D_2 , GABA_A , or GABA_B receptors (Watts et al. 1996; Cathala and Paupardin-Tritsch 1999; Arencibia-Albite et al. 2007).

I_H is sometimes referred to as “pacemaker current,” because it helps to generate rhythmic activity within groups of heart and brain cells. In DA neurons, the inhibition of I_H by Cs^+ produces no effect on spontaneous firing (Mercuri et al. 1995). When treated with the more selective inhibitor ZD7288, a subgroup of SN DA neurons show a decrease in the firing rate (Fig. 3b, Seutin et al. 2001; Neuhoff et al. 2002; Puopolo et al. 2007). However, even in those cells, I_H is not essential for pacemaking, since at concentrations relatively selective for I_H channels, ZD7288 only partially inhibits the firing.

Neuhoff et al. (2002) found that I_H channel density is correlated with the location of the cell and the presence of the calcium-binding protein calbindin. VTA DA neurons projecting the nucleus accumbens (NAc) exhibit significantly smaller I_H than those projecting to the basolateral amygdale (BLA). In both groups of cells, I_H is significantly smaller than in SN DA neurons (Ford et al. 2006). More recently, Lammel et al. (2008) show that DA neurons projecting to the PFC also exhibit little to no I_H . In their studies, however, I_H is not significantly smaller in NAc-projecting neurons than in BLA-projecting cells. Margolis et al. reported that VTA cells that lack I_H are all TH^- . However, a major portion of VTA cells expressing large I_H are TH^- too. These cells are electrophysiologically indistinguishable from DA neurons, since they also exhibit broad action potentials and are hyperpolarized by the D_2 agonist quinpirole (Margolis et al. 2006). Whether these cells correspond to the novel wide-

spike neurons described by Luo et al. (2008) remains to be determined. It is important to point out, however, that the expression of I_H can vary significantly depending on the experimental conditions. Adding 8-Bromo-cAMP to intracellular recording solution, for example, induces an 11 mV shift of the half-activation voltage (Franz et al. 2000). Phosphatidylinositol-4,5-bisphosphate also controls I_H independently of the action of cyclic nucleotides (Zolles et al. 2006). I_H also changes during postnatal development (Walsh et al. 1991; Washio et al. 1999; Chan et al. 2007).

In the SN, I_H was thought to be present in DA and not non-DA neurons (Kita et al. 1986; Nakanishi et al. 1987; Stanford and Lacey 1996; Richards et al. 1997). Recent studies show that I_H is also expressed in non-DA neurons, but it is small compared with that seen in DA neurons (Atherton and Bevan 2005; Lee and Tepper 2007). Similar results have been reported for VTA non-DA neurons (Johnson and North 1992).

Transient Outward Rectification and A-type K^+ Channels

When depolarized from a hyperpolarized level, DA neurons often show a transient outward rectification (Fig. 3a). This rectification slows the depolarization, leading to a delay in spiking (Kita et al. 1986; Grace and Onn 1989). Studies in other cells suggest that the transient outward rectification is mediated by 4-aminopyridine (4-AP)-sensitive, A-type K^+ channels. Early studies in DA cells show, however, that 4-AP is ineffective in inhibiting the transient outward rectification (Grace and Onn 1989; Harris et al. 1989). Recent

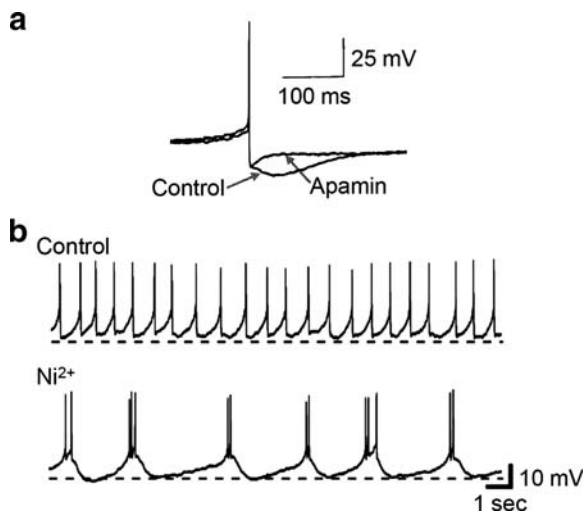


Fig. 4 The spike AHP in DA neurons. (a) Intracellular recordings showing blockade of the AHP in a DA neuron by the SK channel blocker apamin (1 μ M, modified after Shepard and Bunney 1991). (b) Perforated-patch recordings showing that the T-type channel blocker Ni^{2+} (100 μ M) switches the firing pattern of a DA neuron from pacemaker to bursting (modified after Wolfart and Roeper 2002).

studies suggest that 4-AP blocks at least part of the current mediating the transient outward rectification (Liss et al. 2001; Durante et al. 2004). Three subfamilies of voltage-sensitive K^+ channels show the characteristics of A-type channels: Kv1 (Kv1.4), Kv3 (Kv3.3, Kv3.4), and Kv4 (Kv4.1, Kv4.2, and Kv4.3). The SN pars compacta expresses mainly Kv4.3 (Serodio and Rudy 1998). Liss et al. (2001) further show that SN DA neurons express Kv4.3L (long version), but not Kv1.4, Kv3.4, Kv4.1, or Kv4.2, and the expression correlates with the level of I_A . DA cells also express the β subunit KChIP3, which, by interacting with Kv4, increases the current amplitude and alters the gating properties of A-type channels (Liss et al. 2001).

As expected, the blockade of A-type channels by 4-AP or heteropodatoxin, a specific blocker of Kv4.2 and Kv4.3 channels, increases the firing rate of DA neurons (Fig. 3c, Grace 1990; Liss et al. 2001; Ishiwa et al. 2008). This increase in firing is associated with a significant decrease in the action potential threshold (Grace 1990; Nedergaard 1999). A-type channels also influence the shape of action potential (Nedergaard 1999). Thus, the blockade of these channels by 4-AP increases the duration of action potentials and the effect is reversed by the Ca^{2+} channel blocker Cd^{2+} , suggesting that the activation of A-type of channels reduces the ability of an action potential to activate Ca^{2+} channels. 4-AP also has a biphasic effect on the spike afterhyperpolarization (AHP); it reduces the fast component and increases a slow component of AHP. The first effect may result from the blockade of A-type channels that remain open immediately after the action potential. The enhancing effect on the slow component may be secondary to the increased Ca^{2+} influx by

4-AP and the subsequent activation of Ca^{2+} -dependent K^+ channels. Consistent with this possibility, the enhancing effect of 4-AP on the slow AHP is reversed by Cd^{2+} or the SK channel blocker apamin. These effects of 4-AP are more pronounced when action potentials are triggered from a relatively hyperpolarized level, consistent with the fact that A-type channels are activated most effectively from a hyperpolarized potential and are inactivated when the cell is held continuously at a depolarized potential. Computational simulation supports a role for these channels in modulating the shape of action potential in DA neurons (Segev and Korngreen 2007). Simultaneous multisite recordings suggest that somatic A-type channels have a strong inhibitory control over the backpropagation of action potentials from an axon bearing dendrite to the soma and other dendrites (Gentet and Williams 2007).

A-type channels were initially thought to be present only on DA neurons (Richards et al. 1997). Evidence now suggests that they are also expressed in nearby non-DA neurons. However, A-type channels in the two types of neurons are different. First, I_A is much larger in DA neurons than non-DA neurons, which may explain why the delay of the first spike following the termination of a hyperpolarization pulse is much longer in DA neurons than non-DA neurons. Second, both the rate of activation and inactivation are slower in DA neurons compared with non-DA neurons, while the rate of recovery from inactivation is much faster in DA neurons. These differences, together with the fast firing rate of non-DA neurons, suggest that A-type channels in non-DA neurons are not as important as they are in DA neurons for the regulation of spontaneous firing. Third, A-type channels are more sensitive to 4-AP in non-DA neurons than DA neurons. At 1 mM, 4-AP inhibits I_A by 70% in non-DA neurons. The same concentration produces only 24% of inhibition in DA neurons (Koyama and Appel 2006b).

Spike After Hyperpolarization

When fired in a single spike, pacemaker mode, each action potential in DA neurons is followed by a prominent AHP. A major portion of the AHP is mediated by Ca^{2+} -dependent K^+ channel, since it is blocked by the Ca^{2+} -dependent K^+ channel blocker apamin (Fig. 4a, Shepard and Bunney 1988). The AHP is also blocked by removing Ca^{2+} from the extracellular medium or by Ca^{2+} channel blockers such as Co^{2+} (Kita et al. 1986; Yung et al. 1991; Nedergaard et al. 1993). There are three subfamilies of Ca^{2+} -dependent K^+ channels: the small-, intermediate-, and large-conductance channels, termed SK, IK, and BK channels respectively. Apamin selectively blocks SK channels.

Three subtypes of SK channels have been cloned: SK1 ($\text{K}_{\text{Ca}2.1}$), SK2 ($\text{K}_{\text{Ca}2.2}$), and SK3 ($\text{K}_{\text{Ca}2.3}$). They can be

distinguished by their differential sensitivity to apamin, with SK1 channels being the least sensitive, SK2 channels the most sensitive, and SK3 channels presenting an intermediate sensitivity to apamin. In some literatures, the SK family also includes $K_{Ca3.1}$ (SK4) channels, which are insensitive to apamin and are blocked by the scorpion toxin charybdotoxin. DA neurons express mainly SK3 channels, with SK1 and/or SK2 mRNA detected only in a minority of DA cells. There is also little SK3 immunoreactivity in non-DA neurons. SK3 immunoreactivity is about four fold lower in the VTA compared with the SN. Electrophysiologically, VTA DA neurons also possess four fold smaller AHP currents (Wolfart et al. 2001).

SK channels lack an obvious calcium-binding domain. Their Ca^{2+} sensitivity is conferred by calmodulin, which is constitutively bound to the C-terminus of the channel and causes channel opening upon binding of Ca^{2+} . Wolfart and Roeper suggest that Ca^{2+} influx through T-type Ca^{2+} channels is critical to the activation of SK channels in DA neurons, since low doses of Ni^{2+} and mibefradil block not only T-type Ca^{2+} current but also SK-mediated AHP (Fig. 4b). In contrast, inhibitors for L-type and P/Q-type Ca^{2+} channels produce no significant effect on AHP current (Wolfart and Roeper 2002). Nedergaard et al reported, however, that the L-type channel blocker nifedipine also reduced an apamin-sensitive, slow component of the AHP in DA neurons (Nedergaard et al. 1993). N-type Ca^{2+} channels also play a partial role in SK channel activation during the AHP (Nedergaard et al. 1993; Wolfart and Roeper 2002).

Ca^{2+} influx through voltage-gated Ca^{2+} channels can also activate SK channels indirectly by triggering Ca^{2+} release from intracellular Ca^{2+} stores (Cui et al. 2007). In DA neurons, both IP_3 and ryanodine receptors are involved in Ca^{2+} -induced Ca^{2+} release (Morikawa et al. 2003; Cui et al. 2007). In the absence of action potential and the absence of Ca^{2+} entrance through voltage-gated channels, an increase in intracellular IP_3 is enough to activate SK channels by causing Ca^{2+} release from intracellular stores (Morikawa et al. 2000; Cui et al. 2007). This mechanism may underlie the metabotropic glutamate receptor (mGluR)-induced hyperpolarization of DA neurons (Fiorillo and Williams 1998; Morikawa et al. 2003) and be partially responsible for the spontaneous, apamin-sensitive, miniature hyperpolarizations seen in DA neurons from young animals (Seutin et al. 1998; Seutin et al. 2000; Cui et al. 2004). Katayama et al suggest that in young rats, the mGluR-mediated outward current is mediated by IK, but not SK channels, since the current is blocked by charybdotoxin, a blocker of both BK and IK channels, but not by apamin or the BK channel blocker iberiotoxin (Katayama et al. 2003).

The blockade of SK channels by apamin has been reported to produce variable effects or an increase in firing in DA neurons recorded in vitro (Shepard and Bunney 1988, 1991; Wolfart et al. 2001; Kim et al. 2007). The SK channel

activator 1-ethyl-2-benzimidazolinone (1-EBIO) decreases DA cell firing (Wolfart et al. 2001; Kim et al. 2007). The effect of apamin on firing pattern appears to be more consistent; in most DA cells, apamin increases the variability of firing. In a subgroup of DA cells, apamin converts regular spiking into bursting. Systemic administration of apamin (0.4 mg kg^{-1} , i.v.) also increases bursting in approximately 50% of the DA neurons tested and the effect is accompanied by no change in average firing rate (Ji and Shepard 2006). Systemic injection of 1-EBIO ($5\text{--}25 \text{ mg kg}^{-1}$) also has no effect on the average firing rate, but it suppresses bursting activity and increases the precision of firing of DA neurons. SN DA neurons express more SK3 proteins and larger SK currents than VTA DA cells. They also exhibit a more regular firing pattern than VTA DA cells, consistent with a role of SK channels in regulation of firing pattern (Wolfart et al. 2001). Within the VTA, the precision of firing of DA cells is also positively correlated with the amplitude of the apamin-sensitive AHP (Koyama et al. 2005).

SK3 channels are present on DA and not neighboring non-DA neurons (Sarpal et al. 2004). GABA neurons in the SN pars reticulata express mainly SK2 but not SK3 channels (Yanovsky et al. 2005). Consistent with this finding, the AHP in GABA neurons is more sensitive to apamin than in DA neurons. There are two other differences between SK channels in DA and GABA neurons. First, during the AHP, SK channels in GABA neurons are activated by Ca^{2+} influx through not only T-type but also N-type channels. Second, Ca^{2+} release from intracellular store is not involved in the activation of SK channels during the AHP in GABA neurons (Atherton and Bevan 2005; Yanovsky et al. 2005). However, during the spontaneous, action potential-independent, and apamin-sensitive miniature hyperpolarizations, Ca^{2+} release from intracellular store does contribute to the activation of SK channels in GABA neurons (Yanovsky et al. 2005).

Limited evidence suggests that several other types of channels are also activated during the AHP in DA neurons, including the 4-AP sensitive I_A channels (Nedergaard 1999), KCNQ K^+ channels (Scroggs et al. 2001; Koyama and Appel 2006a), and ERG K^+ channels (Nedergaard 2004; Shepard et al. 2007).

Firing Patterns of DA Neurons

In Vivo Studies

Burst Firing

DA neurons have been thought to fire in either single-spike or burst mode. Early observation by Bunney et al. (1973b) suggests that DA, but not non-DA, cells in the VTA and SN

exhibit burst firing. Furthermore, this type of firing pattern is more frequently observed in chloral hydrate-anesthetized animals than in a non-anesthetized, paralyzed preparation. To quantitatively measure the level of bursting, Grace and Bunney (1984) proposed to identify bursts based on interspike intervals (ISIs). Their analysis of visually identified bursts suggests that a pair of spikes with an ISI less than 80 ms usually marks the beginning of a burst. Subsequent spikes are considered to be part of the initial burst until an ISI greater than 160 ms is encountered.

Although the above described “80/160 ms” criteria have been widely used, recent analyses suggest that the criteria have several limitations. First, they tend to split a single “natural” burst into multiple “artificial” bursts (Shi et al. 2004; Shi 2005; Zhang et al. 2008). For example, according to the criteria, the second burst in Fig. 5a, marked by the thick blue bar, consists of not one but three “bursts” (marked by the thin red bars). Second, not all spikes fired in bursts are recognized by the criteria. For example, the sixth spike in the second burst (marked by the blue bar) and the first two spikes in the fourth burst (marked by the thick purple bar) are all

identified as non-burst spikes by the criteria. Third, the criteria do not distinguish bursts from high-frequency, irregular firing. Consequently, in cells firing at a high rate, ISIs within the so-called bursts are not markedly different from ISIs between bursts (Shi 2005; Zhang et al. 2008). In those cells, bursts identified by the criteria tend to also have a duration that would be too long for them to be considered as bursts. These limitations may explain why there is a correlation between the firing rate and the level of bursting in fast firing, but not low-firing, DA cells (Hyland et al. 2002; Robinson et al. 2004). They may also explain the discrepancy between studies regarding the level of bursting in nonanesthetized, paralyzed animals. Early studies suggest that the level of bursting, estimated based on visual and audio inspection, is much lower in nonanesthetized, paralyzed animals than in chloral hydrate-anesthetized rats (Bunney et al. 1973b). When analyzed using the “80/160 ms” criteria, however, the percent of spikes fired in the so-called bursts is significantly higher in nonanesthetized, paralyzed animals compared with chloral hydrate-anesthetized rats (23 ± 3 vs. $15 \pm 3\%$, Kelland et al. 1989). The criteria also classify more DA neurons as bursting cells in nonanesthetized, paralyzed rats than in chloral hydrate-anesthetized animals (72% vs 44%, Kelland et al. 1989).

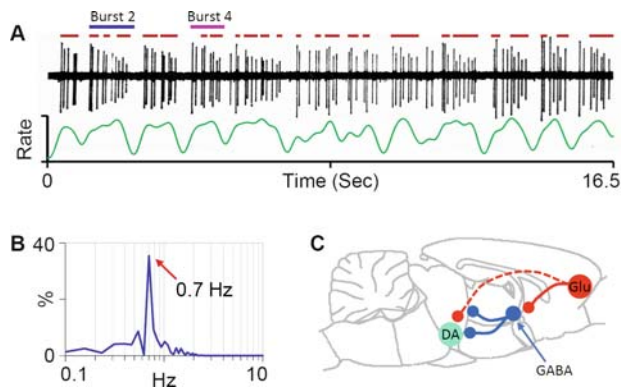


Fig. 5 Firing patterns of DA neurons. (a) The spike train in the middle is the same spike train shown in Fig. 1a. Spikes fired in “bursts”, defined by the “80/160 ms” criteria, are indicated by the red horizontal bars above the spike train. Below the spike train is a smoothed rate histogram (binwidth=50 ms) constructed based on the ISIs measured from the spike train. According to the early description by Bunney et al, the spike train contains approximately 12 bursts. The second and fourth bursts are marked by the thick blue and purple bars, respectively. According to the “80/160 ms” criteria, however, spikes under the blue bar constitute not one, but three separate “bursts” plus one single spike. The criteria also divide the spikes under the purple bar into two single spikes and two “bursts”. These and other observations (Shi 2005) suggest that the bursts defined by the “80/160 ms” criteria are different from the bursts originally described by Bunney et al. The rate histogram further suggests that the firing pattern of the cell can be described as slow oscillations (SO) in firing rate. (b) Spectrum of the rate histogram shown in A confirming the presence of SO. The peak frequency of the SO (0.7 Hz) coincides with the frequency of bursting (7 bursts per 10s). (c) Schematic drawing illustrating possible mechanisms underlying the SO observed in VTA DA neurons. The inverse relation between the SO in DA neurons and that in the PFC suggests that part of PFC information is relayed to DA neurons through inhibitory cells (Gao et al. 2007). The latter may inhibit DA cells directly or modulate excitatory inputs to DA neurons.

Slow Oscillatory Firing

Spectral analysis suggests that the burst firing, originally described by Bunney et al, can be described as slow oscillations (SO) in firing rate (Fig. 5a, b). The frequency of the SO corresponds to the frequency of bursts, while their amplitude is correlated with both the number and frequency of spikes within each burst (Fig. 5b, Shi et al. 2004; Shi 2005; Zhang et al. 2008). Simultaneous multisite recordings suggest that the SO in VTA DA neurons are, at least partially, derived from the PFC, since they are highly coherent with the oscillatory activity in the PFC and inhibited when the PFC is inactivated. Unexpectedly, the SO in most VTA DA neurons exhibit an inverse relation with the activity of PFC neurons (Gao et al. 2007). Since cortical output neurons are excitatory, this finding suggests that at least part of PFC information is relayed to DA cells through inhibitory neurons (Fig. 5c, Gao et al. 2007).

A smaller percent of non-DA neurons in the VTA also display SO in their firing activity. Different from DA neurons, the SO in most non-DA neurons have a nearly in-phase relation with the SO in the PFC, suggesting that PFC input may directly cause the SO in non-DA cells (Gao et al. 2007). DA neurons in the SN also exhibit the SO, but it is much reduced compared with VTA DA neurons (Zhang et al. 2008). Preliminary studies suggest that the SO in SN DA

neurons also depend on synaptic input (Shi, unpublished data), but the source of the input remains to be determined.

In Vitro Studies

In contrast to the different firing patterns observed *in vivo*, DA neurons recorded in brain slices fire in a regular, pacemaker-like fashion, supporting the suggestion that the irregular firing seen *in vivo* is synaptically mediated. A number of studies have tried to reproduce the rhythmic burst firing or the SO in brain slices by tonically activating or inactivating certain receptors or channels. Shepard and Bunney were the first to show that the blockade of the SK channels by apamin induces irregular firing and, in some DA cells, bursting (Shepard and Bunney 1988). Johnson et al showed that the prolonged activation of NMDA receptors, particularly in the presence of the SK channel blocker apamin, induces rhythmic burst-like activity (Johnson et al. 1992). Kitai et al proposed that the activation of muscarinic receptors may mimic the effect of apamin, enhancing the ability of NMDA to induce bursting (Kitai et al. 1999; Scroggs et al. 2001). In the presence of SK channel blockers, the activation of Group I mGluRs has also been shown to induce bursting in DA neurons (Prisco et al. 2002). More recently, Chen and colleagues found that carbachol, which activates both nicotinic and muscarinic receptors, induces rhythmic bursting in about 20% of DA neurons tested. Their data further show that the effect is mediated through opening of L-type of Ca^{2+} channels and involves the activation of protein kinase M (Zhang et al. 2005; Liu et al. 2007).

However, bursts induced *in vitro* tend to have temporal characteristics different from those observed *in vivo*. In most studies, the number of spikes per burst is significantly higher than that observed *in vivo*. The frequency of bursts also tends to be low. These differences point to the possibility that the rhythmic bursting or the SO seen *in vivo* are not caused by a tonic activation or inactivation of specific receptors or channels, but are due to oscillatory or phasic synaptic input (Gao et al. 2007). Blythe et al recently show that DA cells *in vitro* are capable of generating bursts similar to those observed *in vivo* when they are stimulated by transient, high-frequency glutamate synaptic inputs or by a brief application of glutamate to DA cell dendrites. Both AMPA and NMDA receptors are involved in the effect (Blythe et al. 2007).

Feedback Control of DA Neurons

After its release, DA affects not only cells postsynaptic to DA terminals but also DA neurons themselves through various feedback pathways. Bunney et al provided the first

electrophysiological evidence for the feedback regulation of DA neurons (Bunney et al. 1973b). Their studies show that DA antagonists increase the firing of DA cells and the effect is more pronounced in locally anesthetized, paralyzed animals than in chloral hydrate-anesthetized rats. d-Amphetamine, on the other hand, inhibits DA neurons and the inhibition is blocked by DA antagonists as well as the DA synthesis inhibitor α -methyl tyrosine, and is mimicked by the indirect DA agonist l-dopa and the direct DA agonist apomorphine (Bunney et al. 1973a). Studies with d-amphetamine further show that there are two types of feedback pathways: a short one mediated by DA autoreceptors on DA neurons and long ones involving DA target neurons that project back to DA cells either directly or indirectly (Bunney and Aghajanian 1975; Bunney and Aghajanian 1976; Bunney and Aghajanian 1977, 1978). The DA agonist-induced inhibition has been used as part of the criteria for the identification of DA cells, since it is observed only in DA cells and not neighboring non-DA neurons. However, as discussed in Section 1, some VTA non-DA neurons are reported to be also inhibited by DA agonists, and a subgroup of DA neurons projecting to the PFC has been suggested to lack the inhibitory DA autoreceptor.

DA Autoreceptors

Aghajanian and Bunney provided the first evidence for the presence of DA autoreceptors on DA cell soma and dendrites (Aghajanian and Bunney 1977a, b). Studies in brain slices further show that the activation of these receptors causes a hyperpolarization of the cell (Pinnock 1984; Lacey et al. 1987, 1988; Silva and Bunney 1988). Several lines of evidence suggest that DA autoreceptors mediate the inhibition of DA neurons induced by low doses of systemically administered apomorphine. Thus, the inhibition is largely unaltered by lesions of forebrain inputs to DA neurons, suggesting that forebrain inputs contribute minimally to the effect (Aghajanian and Bunney 1974). Consistent with this suggestion, low doses of apomorphine that produce a significant inhibition of DA neurons have only a limited effect or no effect on DA target neurons in the striatum (Skirboll et al. 1979). A role for DA autoreceptors in the effect of apomorphine is further supported by the finding that the effect is greatly reduced after DA autoreceptors are inactivated by the local infusion of pertussis toxin (Innis and Aghajanian 1987).

There are two subfamilies of DA receptors: D1- and D2-like. The D1-like family contains D_1 and D_5 receptors and the D2-like family consists of D_2 , D_3 , and D_4 receptors. The D_2 receptor has two isoforms: long ($\text{D}_{2\text{L}}$) and short ($\text{D}_{2\text{S}}$), which differ by the presence or absence of 29 amino acids in

the third cytoplasmic loop. D1-like receptors are coupled G_s , whereas D2-like receptors are coupled to $G_{i/o}$. DA autoreceptors are D2-like receptors, since the inhibition of DA neurons induced by low doses of apomorphine is mimicked by D2 agonists and blocked by D2 antagonists (Mereu et al. 1985; Carlson et al. 1986; Napier et al. 1986; Carlson et al. 1987; Kelland et al. 1988; Huang and Walters 1992). The effect of apomorphine is also blocked by pertussis toxin, which selectively inactivates G_i and G_o proteins (Innis and Aghajanian 1987). Studies in brain slices confirm that stimulation of D2- but not D1-like receptors hyperpolarizes DA neurons (Lacey et al. 1987, 1988).

DA neurons express both D₂ and D₃ receptors (Bouthenet et al. 1991; Meador-Woodruff and Mansour 1991; Diaz et al. 2000). Studies with D₂ or D₃ receptor-knockout mice suggest that the DA-induced hyperpolarization depends on the activation of D₂, but not D₃, receptors (Mercuri et al. 1997; Centonze et al. 2002; Davila et al. 2003; Beckstead et al. 2004). Since the effect persists in DA cells lacking only D_{2L} receptors, it is further suggested that D_{2S}, but not D_{2L}, receptors are responsible for the effect of DA (Centonze et al. 2002). However, it is unknown whether genetic deletion of D_{2L} receptors alters the functional expression of D_{2S} receptors. It also remains to be determined whether selective D_{2S} deletion can eliminate the hyperpolarizing effect of DA.

Contrary to studies using knockout mice, recordings in wild-type animals show that the potencies of DA agonists to inhibit DA neurons are significantly correlated with their affinities for D₃, but not D_{2L} receptors (Kreiss et al. 1995). Since the affinity of a DA agonist for D_{2S} receptors is similar to its affinity for D_{2L} receptors (Leysen et al. 1993), the above finding suggests that systemically administered DA agonists act predominantly through D₃ receptors to inhibit DA neurons. This suggestion is supported by several other *in vivo* studies in wild-type rats (Lejeune and Millan 1995; Piercey et al. 1996; Wicke and Garcia-Ladona 2001). However, in D₃ receptor-knockout mice, the putative D₃ agonist PD128907 was found to still inhibit DA neurons and its potency was not reduced compared with wild-type animals (Koeltzow et al. 1998). The reason for the discrepancy is unknown. It is possible that there is an interaction between D₂ and D₃ autoreceptors. In normal DA neurons expressing both receptors, the activation of D₃ receptors contributes to the DA-mediated inhibition. In DA cells with the D₂ receptor gene deleted, the stimulation of D₃ receptors becomes ineffective. The deletion of D₃ receptors, on the other hand, may enhance the affinity of D₂ receptors for D₃ agonists.

Studies *in vitro* suggest that DA autoreceptor activation increases the membrane conductance to K^+ (Lacey et al. 1987, 1988). Recordings *in vivo* show, however, that the apomorphine-induced hyperpolarization is associated with a decrease in membrane conductance (Grace and Bunney 1985). Although the latter effect has been suggested

to be indirectly mediated by long feedback pathways (Grace and Bunney 1985), a recent study in brain slices shows that DA, released endogenously following a single action potential, hyperpolarizes neighboring DA neurons by inhibiting I_H channels (Vandecasteele et al. 2008). Thus, it is possible that DA autoreceptors are linked to different effector systems. Receptors that are coupled to I_H channels may be preferentially located in the vicinity of DA release sites, whereas those coupled to G-protein-gated K^+ channels may be extrasynaptic. Studies in cultured cells suggest that DA autoreceptors also regulate the functional expression of I_A channels through the cAMP-PKA pathway (Hahn et al. 2003; Hahn et al. 2006).

Long Feedback Pathways

The electrophysiological evidence for the presence of long feedback pathways comes largely from studies with d-amphetamine. Like direct D2 agonists such as apomorphine, d-amphetamine, given systemically, inhibits DA neurons. The inhibition induced by d-amphetamine, however, is reversed by not only DA antagonists but also the GABA antagonist picrotoxin. Lesions of the striatum also significantly reduce the inhibitory effect of d-amphetamine, suggesting that the effect is partially mediated by DA receptors on DA target neurons in the striatum (Bunney and Aghajanian 1975, 1976, 1977, 1978). The activation of these receptors may inhibit DA neurons by increasing GABA release from striatal terminals that innervate DA neurons. Consistent with this possibility, doses of d-amphetamine that produce a significant effect on DA neurons also affect DA target cells, including those in the striatum (Bergstrom and Walters 1981; Kamata and Rebec 1983). Since low doses of d-amphetamine preferentially increase DA release in DA target areas (Kalivas and Duffy 1991), the inhibition of DA neurons induced by those doses of d-amphetamine may be primarily mediated through long feedback pathways. Part of the inhibition of VTA DA neurons induced by cocaine has also been suggested to be mediated by long feedback pathways (Einhorn et al. 1988).

D1- and D2-like receptors are unevenly distributed in the striatum. Neurons that project directly to the SN express mainly D1-like receptors. Those giving rise to the indirect striatonigral pathway express mainly D2-like receptors (e.g., Gerfen 1984, 1985; Yung et al. 1995). A similar pattern of DA receptor expression is observed in the nucleus accumbens (Lu et al. 1997; Lu et al. 1998). Although these observations suggest that both D1- and D2-like receptors play a role in the feedback control of DA neurons, earlier studies show that the selective activation of D1-like receptors produces no effect or an inconsistent effect on DA cells

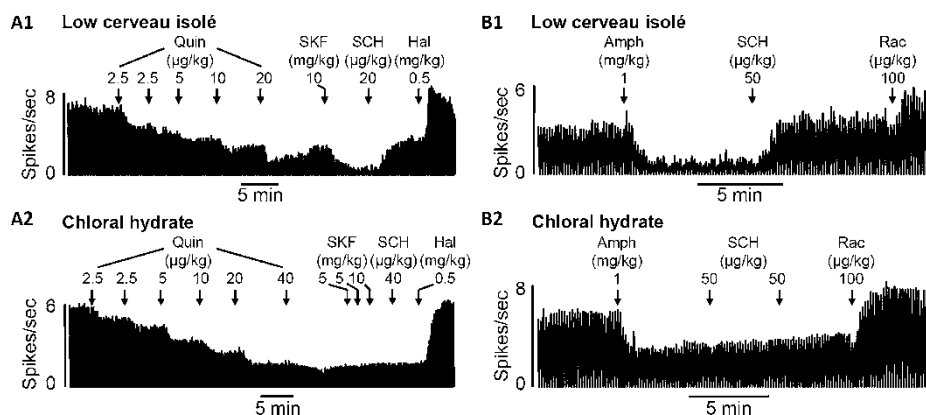


Fig. 6 Feedback control of DA neurons and the role of D1 and D2-like receptors. (a) Rate histograms (binwidth=10 sec) of two different DA neurons recorded, respectively, from a low cerveau isolé (A1) and chloral hydrate-anesthetized rat (A2). The D2 antagonist quinpirole (Quin) decreases the firing in both cells. In the low cerveau isolé rat, the D1 agonist SKF38393 (SKF), given after quinpirole (cumulative dose: 40 $\mu\text{g/kg}$), produces a further inhibition. This inhibitory effect of SKF38393 is not observed in rats treated with vehicle or only a low dose of quinpirole ($\leq 20 \mu\text{g kg}^{-1}$, data not shown). The D1 antagonist SCH23390 (SCH) selectively reverses the inhibition induced by SKF38393. Haloperidol (Hal) reverses the remaining inhibition. In the chloral hydrate-anesthetized rat, SKF38393, given after quinpirole, produces no effect on the cell. Subsequent injection of SCH23390 also produces no effect. Haloperidol completely reverses the inhibition induced by quinpirole (modified after Shi et al. 1997). (b) Rate histograms of two other DA neurons, one recorded from a low cerveau isolé rat (B1) and one from a chloral hydrate-anesthetized rat (B2). In both cells, d-amphetamine (Amph) inhibits the firing. In the low cerveau isolé rat, SCH23390 reverses the inhibition induced by d-amphetamine. Raclopride (Rac) further increases the firing rate to above baseline. In the chloral hydrate-anesthetized rat, SCH23390 produces no effect on d-amphetamine-induced inhibition. Subsequent injection of raclopride reverses the inhibition and increases the firing rate to above baseline (modified after Shi et al. 2000a). These results, together with those discussed in the text, suggest that both D1 and D2-like receptors are involved in feedback control of DA neurons. However, the expression of the D1-like receptor effect requires co-activation of D2-like receptors and is inhibited by chloral hydrate anesthesia.

and the inhibition induced by apomorphine is reversed by D2 but not D1-like receptor antagonists (Mereu et al. 1985; Carlson et al. 1986; Napier et al. 1986; Carlson et al. 1987; Kelland et al. 1988; Huang and Walters 1992). In one study, however, the inhibition induced by high doses of apomorphine is slightly attenuated by the D1 antagonist SCH23390 (Napier et al. 1986).

We hypothesized that D1-like receptors are involved in the feedback regulation of DA neurons. The expression of their effect, however, requires the coactivation of D2-like receptors on DA target neurons (Shi et al. 1997; Shi et al. 2000a). The hypothesis is based on the observation that the expression of some DA effects require concurrent activation of D1 and D2-like receptors (e.g., Walters et al. 1987; White 1987; Bordi and Meller 1989; Wachtel et al. 1989; Bertorello et al. 1990). We tested the hypothesis using a locally anesthetized, paralyzed rat preparation (low cerveau isolé preparation), because general anesthesia has been shown to block or reduce the effect of DA agonists on DA target neurons (Bergstrom et al. 1984). Supporting the hypothesis, D1-like receptor agonists consistently inhibit DA cells when the rat is pretreated with a high dose of the D2-like receptor agonist quinpirole (Fig. 6a1). In animals with DA autoreceptors blocked by the local infusion of raclopride, high doses of quinpirole also enable D1-like receptor agonists to inhibit DA neurons. As expected, chloral hydrate, an anesthetic frequently used in previous studies, blocks the

D1-like receptor-mediated effect (Fig. 6a2, Shi et al. 1997). These findings not only support the presence of multiple feedback pathways suggested by anatomical studies, but also show an interaction between these pathways. This interaction may allow integration of feedback information from the direct and indirect pathways of the basal ganglia and, thus, enable DA neurons to regulate these pathways in a coordinated fashion.

Studies with d-amphetamine confirm that endogenously released DA acts through both D1- and D2-like receptors to inhibit DA neurons (Shi et al. 2000a). Thus, in low cerveau isolé, but not chloral hydrate-anesthetized rats, the D1 antagonist SCH23390 partially or completely reverses the inhibition induced by d-amphetamine (Fig. 6b1, b2). The remaining inhibition is reversed by the D2 antagonist raclopride. Supporting the notion that the D1-like receptor-mediated effect depends on the coactivation of D2-like receptors, raclopride, given before SCH23390, completely reverses d-amphetamine-induced inhibition. Thus, in low cerveau isolé rats, a major portion of the inhibition induced by d-amphetamine is sensitive to the blockade of either D1- or D2-like receptors.

Our studies with d-amphetamine also led to the discovery of a non-DA mediated, excitatory effect of d-amphetamine on DA neurons (Fig. 7, Shi et al. 2000b; Shi et al. 2007). The effect, expressed as an increase in both the firing rate and SO, is largely masked by the DA-mediated inhibition under

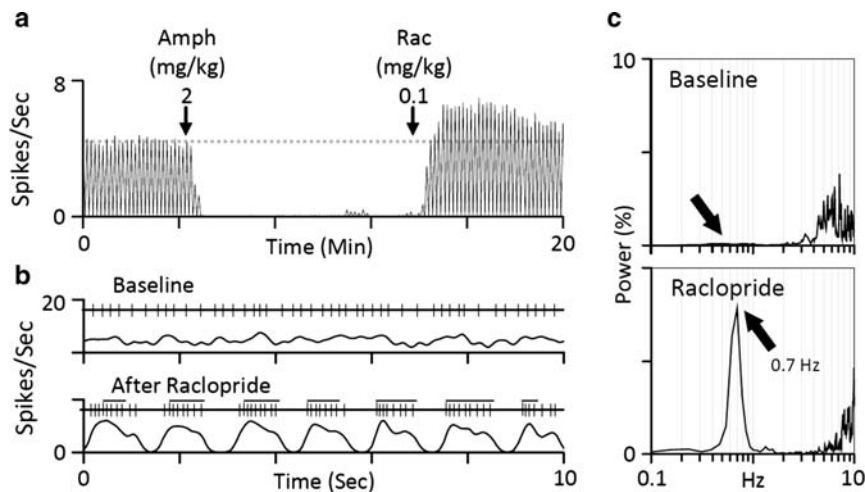


Fig. 7 Multiple effects of d-amphetamine on DA neurons. (a) Rate histogram (binwidth=10 s) of a DA cell showing that the D2 antagonist raclopride (Rac) not only reverses the inhibition induced by d-amphetamine (Amph), but further increases the firing rate to above baseline. (b) Segments of spike trains and corresponding smoothed rate histograms (binwidth=50 ms) showing that raclopride injection also leads to an increase in rhythmic bursting or SO in firing rate. Bursts, defined by the “80/160 ms” criteria, are marked by the horizontal bars above the spike train. (c) Spectra of the rate histograms confirming the increase in SO (arrow) following raclopride injection (from Shi et al. 2007). Additional data, not shown here, suggest that the increase in both firing rate and SO after raclopride injection is an effect of d-amphetamine mediated by non-DA receptors. The effect is normally masked by the DA-mediated inhibition and revealed when the inhibition is blocked by raclopride (see text for further discussion)

control conditions and more clearly observed when the DA-mediated inhibition is attenuated or blocked by a DA antagonist (Zhou et al. 2006). The effect is mimicked by all psychostimulants tested including cocaine, but not by l-dopa and apomorphine, and involves an increase in norepinephrine release and the activation of $\alpha 1$ receptors (Shi et al. 2000b; Shi et al. 2004). The increase in SO induced by psychostimulants suggests that these drugs induce not just an increase in DA release, but a pattern of DA release that is coordinated, on a subsecond scale, with glutamate release from prefrontal terminals (Gao et al. 2007; Shi et al. 2007).

Summary

Since the first electrophysiological study of DA neurons was published 35 years ago (Bunney et al. 1973b), tremendous efforts have been made to understand this small group of neurons in the brain. Current evidence suggests that DA neurons differ from neighboring non-DA neurons in not only their ability to synthesize and release DA but also their electrophysiological properties. The latter define the way DA neurons process synaptic input and determine how the processed information is ultimately delivered to the DA terminal to cause DA release. Using a multidisciplinary approach, studies have begun to uncover the molecular basis underlying the differences between DA and non-DA neurons. Using a similar approach, studies have also shown that not all DA neurons share the same electrophysiological characteristics and that some of the electrophysiological properties of DA neurons are correlated with their anatomical location, projec-

tion site, and other molecular markers of the cell. A better understanding of the heterogeneity of DA neurons may offer critical information for the development of more selective therapeutic interventions for different disorders.

In the last few years, significant advances have also been made in our understanding of the firing patterns of DA neurons. Analyses show that the bursts identified by the widely used “80/160 ms” criteria overlap only partially with those originally described by Bunney et al. This mismatch urges a cautious interpretation of results obtained using the “80/160 ms” criteria. Simultaneous multisite recordings suggest that in chloral hydrate-anesthetized rats, the rhythmic burst-like activity in VTA DA neurons is caused by oscillatory input derived, at least partially, from the PFC and that part of PFC information is transferred to DA cells indirectly through inhibitory neurons.

Early studies by Bunney et al show that DA agonists inhibit DA neurons through both DA autoreceptors and long-loop feedback pathways. New evidence suggests that DA autoreceptors are coupled to multiple effector systems and that both D1- and D2-like receptors are involved in long-loop feedback regulation of DA neurons. This line of research further led to the discovery that psychostimulants including d-amphetamine affect DA neurons through not only DA but also non-DA receptors. The non-DA receptor-mediated effect is not mimicked by l-dopa or apomorphine, and thus, may play an important role in behavioral effects unique to psychostimulants.

Conflicts of interest statement I declare that I have no conflict of interest.

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Chapter 9

Chaotic Versus Stochastic Dynamics: A Critical Look at the Evidence for Nonlinear Sequence Dependent Structure in Dopamine Neurons

C.C. Canavier and P.D. Shepard

Abstract The firing pattern of midbrain dopamine neurons is thought to have important behavioral consequences. Although these neurons fire regularly in vitro when deprived of their afferent inputs, they usually fire irregularly in vivo. It is not known whether the irregularity is functionally important and whether it derives from the intrinsic properties of dopamine neurons or network interactions. It is also not known whether the irregular firing pattern is fundamentally stochastic or deterministic in nature. Distinguishing between the deterministic nonlinear structure associated with chaos and other sources of structure including correlated noise is an inherently nontrivial problem. Here we explain the geometric tools provided by the field of nonlinear dynamics and their application to the analysis of interspike interval (ISI) data from midbrain dopamine neurons. One study failed to find strong evidence of nonlinear determinism, but others have identified such a structure and correlated it with network interactions.

Keywords Chaos • Correlation dimension • Forecasting • Nonlinear dynamics

Introduction

Chaos (Strogatz 2000) is a mathematical term denoting activity patterns that appear to fluctuate randomly and that arise from a sensitive dependence on initial conditions in a completely deterministic system, meaning that there is

no noise or probabilistic component to the describing equations. The interest in chaotic firing patterns in dopamine neurons stems from a theory (King et al. 1984) that the equations that describe a model of the firing rate of dopamine neurons in the intact basal ganglia can enter chaotic regimes based upon the synthesis, effectiveness, and availability of dopamine at postsynaptic sites in the striatum. The major influences on the firing rate in this model were the level of external depolarizing input, the short inhibitory feedback loop mediated by the local release of dopamine within the nigra acting at rate-modulating autoreceptors, and the long feedback loop mediated by feedback GABAergic inhibition from the striatum. A major nonlinearity was introduced into the system via the U-shaped dependency of dopamine synthesis in striatal terminals upon impulse-dependent dopamine release, such that either decreases or increases in the firing rate could increase synthesis. The complexity of the system was increased by a long (20–30 min) delay between changes in dopamine firing rate and changes in dopamine synthesis in the presynaptic terminals in the striatum. Chaotic dopamine neurodynamics (King et al. 1984) were postulated to be responsible for the reported fluctuations in mood, attention, and activity in patients with schizophrenia, including delusions (Shaner 1999) as well as for the “On-Off” phenomenon in Parkinson’s disease.

In the King model, chaos was postulated to arise substantially from network influences on the firing rate of dopamine neurons, not from the intrinsic currents in dopamine neurons themselves, and was postulated to be detrimental to the contingent reinforcement attributed to dopamine signaling. However, dopamine neurons are capable of intrinsic pacemaking (Fujimura and Matsuda 1989; Grace and Onn 1989; Harris et al. 1989; Kang and Kitai 1993; Yung et al. 1991) and are also capable of bursting in the presence of synaptic input in vivo (Grace and Bunney 1984b; Freeman et al. 1985) or pharmacological manipulations in vitro (Johnson et al. 1992; Ping and Shepard 1996). Single neurons that can exhibit both bursting and pacemaking are also capable of

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generating chaotic firing patterns without network involvement (Canavier et al. 1990; Canavier et al. 1993). Furthermore, it has been suggested that rather than being detrimental to certain computations, complex system dynamics on the edge of chaos may actually enhance them (Bertschinger and Natschläger 2004). Distinguishing between chaos and stochastic dynamics in electrophysiological data is difficult for two reasons. The first is that real time series are always contaminated by noise, and hence there is an inevitable stochastic contribution. Second, in an electrophysiological context, the information transmitted to downstream regions in general does not consist of a continuous time series, but rather of series of inter-event intervals called inter-spike intervals (ISIs) between action potentials.

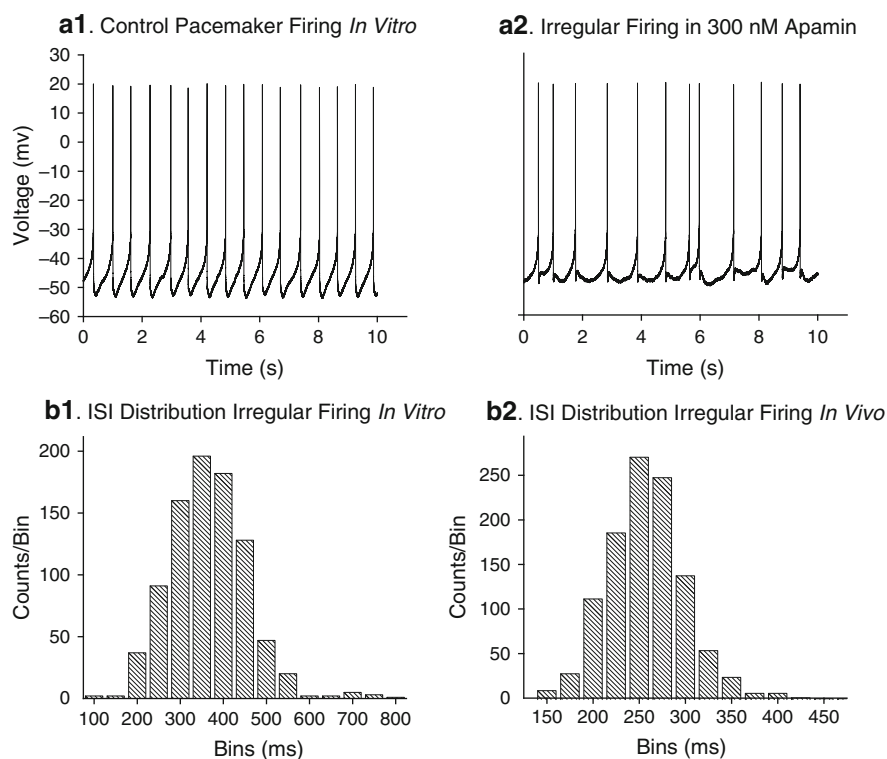
In vivo dopamine neurons can fire in a single spike mode or a bursting mode (Grace and Bunney 1984a, 1984b), but generally fire in a regular pacemaker-like pattern in vitro (Fig. 1a1). The application of the small conductance (SK) potassium channel blocker apamin to dopamine neurons in a slice preparation (Fig. 1a2) can cause them to fire less regularly (Ping and Shepard 1996), approximating the firing pattern in vivo more closely (Fig. 1b). Here, we examine the evidence for nonlinear sequence dependence of these intervals of dopamine neurons both in relative isolation and within a network context.

Methodological Approaches and Considerations

Dynamical Systems and Attractor Reconstruction

A dynamical system (Strogatz 2000) is one that changes in time. A deterministic dynamic system is completely specified by a set of state variables. Membrane potential is an example of a state variable for a neuron, but there are others that can be more difficult to observe, such as ionic concentrations or fractional activation or inactivation of ion channels. Each variable has an associated equation for the rate of change of that variable; the rate of change is measurable as the slope of a plot of the variable vs. time. By definition, the rate of change of a variable can only depend on its own state and that of the other state variables. In all deterministic dynamical systems, the entire future time course of a solution can be completely and uniquely determined by specifying a value for each of the state variables; these values are termed the initial conditions. In a linear system, the rate of change can only depend on the sum of the state variables, each scaled by a constant. The solution trajectories for each state variable are limited to some combination of exponentials with possibly complex exponents such that the solutions either decay to a fixed point at the origin or blow up to infinity or produce oscillations

Fig. 1 Irregular firing in vivo can be approximated in vitro by bath application of apamin (a) Intracellular membrane potential recordings from substantia nigra dopamine neurons in a slice preparation. (a1) Control. (a2) Bath application of 300 nM apamin induces irregular firing. (b) ISI histograms. (b1) Distribution of interspike intervals in an apamin treated neuron in vitro. (b2) Distribution of interspike intervals in an irregularly-firing dopamine neuron recorded extracellularly in vivo. Adapted from Canavier et al. 2004



like sine waves. In a nonlinear system, the form of the dependencies of the rates of change of the state variables on other state variables is not constrained, and the possible solutions are therefore much richer. These solutions include a chaotic attractor in the state space in which the time course of the future trajectories on nearby points locally diverge exponentially from each other. However, the trajectories do not leave the attractor surface, which results in a complex fractal structure for the attractor and a sensitive dependence of the solution trajectories on initial conditions, which are the hallmarks of chaos. The fact that nearby initial conditions result in future time courses that are very different limits the predictability of the system. After the specification of initial conditions, the time window over which a prediction can be made that is better than simply using the mean value of the time series as a prediction is called the short-term prediction horizon. In a chaotic system, the accuracy of the best possible prediction falls off exponentially in time. This follows because the state of a system can only be known with finite precision, and even slightly different initial conditions produce very different solutions after enough time elapses.

A time series is a series of observations of a state variable of the system. Given a stationary time series that is sufficiently long and sufficiently noise-free, an analog of the geometric attractor on which the dynamics in the full state space of the original system reside can be reconstructed (Packard et al. 1980) in a state space formed entirely from observations of a single variable by creating points in a new state space in which the first coordinate is the observation itself and the remaining coordinates are delayed versions of an observation in the time series. The delay is called the lag, and the number of points is the embedding dimension. The lag is usually determined by the first zero of the autocorrelation function or of the mutual information (Kantz and Schreiber 1999). Each successive coordinate is the observation one additional time lag into the past. The embedding dimension is determined by trial and error such that increasing it by one does not significantly affect the results. In other words, points that are nearby at a low value of the embedding dimension may no longer be neighbors as the embedding dimension is increased, which indicates that the estimate of the embedding dimension was initially too low. If the embedding dimension is increased beyond the optimum, the quality of the attractor (and the resulting nonlinear forecasting, see the following section) also degrades in a noisy environment (Yunfan et al. 1998).

Correlation Dimension

Given a reconstructed attractor, the correlation sum is the number of points that fall within a certain radius of each

point, summed over all points and divided by the possible number of pairs of points. The slope of the logarithm of the sum as a function of the logarithm of the radius gives an estimate of the correlation dimension. If a three-dimensional object, for example, is sampled at scales that are small relative to the object such that the balls of radius r generally fall within the object, the correlation sum should be proportional to the volume at a given radius. Since the volume is proportional to r^3 , the logarithm of the sum should scale as $3 \log r$, and hence the estimate of dimension should be approximately 3, which conforms to our intuition. The slope will be invariant only in a certain range of length scales, because very large scales sample regions that are not confined to the attractor and very small scales may be smaller than the resolution between points and also sample the inherent measurement noise. For a chaotic attractor, the dimension will be fractal rather than integral, but may be low-dimensional despite the apparent complexity. On the other hand, purely stochastic, or noisy, system will be infinite dimensional. One caveat is that the correlation dimension is best suited to quantify self similarity, or fractal dimension, when self similarity is known to be presented, and is much less suited to the task of establishing the low dimensionality of a data set (Kantz and Schreiber 1999). This measure is very sensitive to nonstationarity in the data and to insufficient sampling. Another weakness is that points that are nearby in time are also nearby in the state space, but not as a result of geometrical structure but simply due to temporal contiguity, thus these points must be excluded from the correlation sum (Theiler 1986). This is not done commonly in practice, casting doubt upon the validity of some published results. In addition, the range of values of r for which the slope of the correlation sum as a function of the radius is constant should be invariant across a range of embedding dimensions above a minimum dimension to make a convincing case that the estimate is reasonable (Kantz and Schreiber 1999). The presence of noise can destroy the stable plateau of the scaling region in which the slope is invariant (Yunfan et al. 1998). Furthermore, filtered noise can be incorrectly identified as low-dimensional based on the correlation dimension (Osborne and Provenzale 1989; Rapp 1993; Rapp et al. 1993). Therefore, additional methods are required to establish the presence of chaos or low dimensional nonlinear structure.

Nonlinear Forecasting

A geometric attractor that has been reconstructed as described earlier can also be used to exploit the nonlinear dynamics inherent in the time series to forecast the evolution of the time series at each point into the future (Farmer and

Sidorowich 1979). The data can be divided in two parts, such that one part of the data are used to construct the attractor and the other part is used to test the accuracy of the forecasting method. At a minimum, the point to be predicted and points that are close in time to it should be excluded from the prediction. The data that were not used to construct the attractor are also used to construct points with coordinates determined by the time lag and embedding dimension. For each point in the test set, the nearest neighbors on the reconstructed attractor are identified and the weighted average of each point in their known future trajectories is used to estimate the future trajectories of the test points. Then the correlation coefficient between the actual and predicted future observation is used to quantify the accuracy of the prediction. In the studies described in this chapter that generated forecasts using the ISI series directly, an embedding dimension of 4 and a time lag of 1 was used in all cases unless otherwise noted.

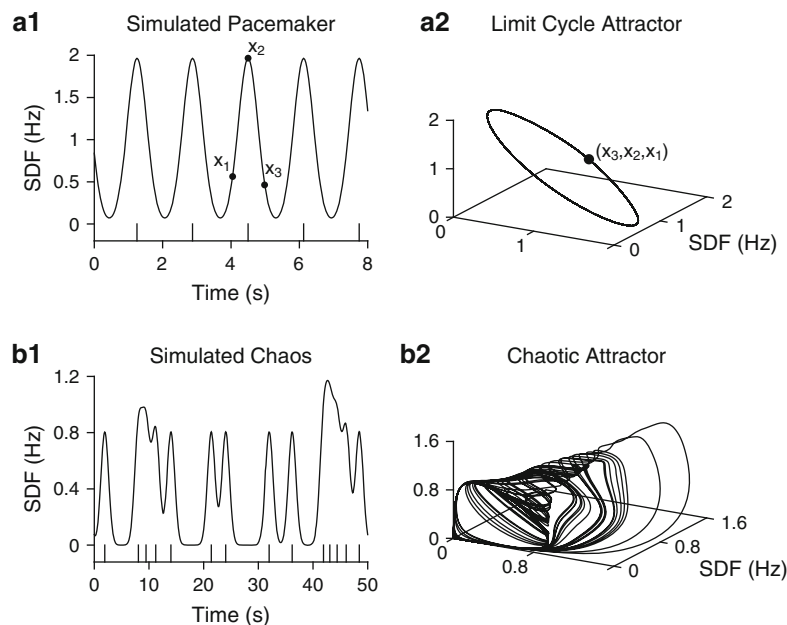
ISI Series Vs. A Time Series

An ISI series is not strictly a time series. ISI series from dopamine neurons are generated using a threshold crossing model; the ISIs are the intervals between the times that the membrane potential crosses a threshold in membrane potential from below. The time intervals between threshold crossings are not sufficient to reconstruct the full attractor, but may be sufficient to reconstruct a rough analogy to a Poincare section with a dimension one less than the original attractor

(Castro and Sauer 1997; Hegger and Kantz 1997). The application of attractor reconstruction methods that were developed for time series to ISI data is only valid when the ISIs are shorter than the short-term prediction horizon (Racicot and Longtin 1997) and when the threshold used to generate the interspike interval times captures the relevant time scales of the underlying dynamics (but see Kaplan et al. 1996 for a case when subthreshold dynamics were essential). This caveat also applies to the use of the correlation dimension and nonlinear forecasting methods that are based on attractor reconstruction. Several studies that address the feasibility of attractor reconstruction from an ISI series did not address the threshold crossing mechanism for generating ISIs, but rather attempted to reconstruct an input signal to an integrate and fire model (Sauer 1994, 1997), which is a distinct problem.

Lovejoy et al. (2001) and Canavier et al. (2004) generated an approximate time series called a spike density function (SDF) from the ISI sequence by superimposing a Gaussian window at each spike time (see Fig. 2a). The purpose of this manipulation was to obtain a time series to determine whether its predictability fell off exponentially as would be expected of a chaotic time series. This exponential falloff would not be expected to be observable in an ISI series unless all ISIs were of similar length such that the ISI number index is a reliable measure of time. In Figure 2 a noiseless model system is used (Plant and Kim 1976) to illustrate the approximate time series recovered from the spike train, seen as a comb plot on the y-axis, for a repetitively spiking pacemaker regime (Fig. 2a1) and a known chaotic regime (Fig. 2b1). The top trace illustrates the mechanics of attractor reconstruction for the pacemaker. The time sequence of the points labeled x_1 , x_2 , and x_3 is shown

Fig. 2 Time series (SDF) generation from an ISI Sequence and attractor reconstruction from a time series. **(a)** Model pacemaker neuron. The spike times are shown as a comb plot on the x-axis in a1. The solid curve in a1 is the spike density function (SDF) created by superimposing a Gaussian window at each spike time. The three points (x_1, x_2, x_3) in a1 are separated by a time lag, and are used as the coordinates to create the labeled point on the limit cycle attractor in a2. **(b)** Model chaotic neuron. The same procedure was used to create the more complex waveform for the spike train shown in a1, and the coordinates each point in the attractor in b2 was created from a set of time lagged points in b1 as in the example shown in a. Adapted from Lovejoy et al. (2001)



in Fig. 2a1 and the corresponding point on the simple geometric attractor corresponding to repetitive periodic activity is shown in Fig. 2a2. The more complex waveform for simulated chaos is shown in Fig. 2b1 and the corresponding more complex attractor is shown in Fig. 2b2.

Surrogate Data

The key to determining whether a time series or ISI sequence contains dynamic nonlinear structure is to isolate the amount of predictability that is due to nonlinear dynamics and not to linear structure (autocorrelations) or static nonlinearities. This is accomplished by manipulating the original data to produce surrogate data sets that preserve the linear structure as well as any static nonlinearities, but destroy any nonlinear dynamical structure. The null hypothesis is that the time series (or ISI series) was produced by linear system with additive noise and that the amplitude was then scaled nonlinearly to produce the observed time (or ISI) series. This nonlinearity is called static because it is not dependent on the temporal history, but only on the instantaneous value of the underlying linear system. These surrogates are known as Gaussian surrogates (Thieler et al. 1992) and are produced as follows. First a set of Gaussian deviates are generated and arranged in the same rank order as the original data series. Then the Fourier transform is applied to the rank-ordered Gaussian deviates. The phase of the transform is randomized, then the inverse transform of the phase-randomized transform is computed. The original data are then shuffled so that its rank order corresponds to that of the inverse transform of the phase-randomized Gaussian deviates. This preserves the underlying linear autocorrelation structure of the data as well as any static nonlinearities, but destroys any sequence-dependent information. Any statistical analysis of whether predictability is due to nonlinear dynamical structure must arise from a comparison of the predictability of the actual data and surrogate data that control for predictability due to other sources such as nonlinear structure.

The surrogate data can be used to estimate a z-score (Thieler et al. 1992) $(Q - \mu)/\sigma$ for an observable (Q) by using the mean (μ) and standard deviation (σ) of the surrogates, which can be used to find the p values under an assumption of Gaussian distribution, or more rigorously, using a nonparametric method to determine the distribution of the statistic nonlinearity in the surrogate data. There are potential problems with this type of surrogate data, such as end effects (Schreiber and Schmitz 2000) and mismatches between discrete frequencies in a Fourier series and the fundamental frequencies of nearly periodic time series (Stam et al. 1998). Another type of surrogate data that

preserve both linear and static nonlinear structure is simply to reverse the order of the time series or sequence (Stam et al. 1998), but this one manipulation is not sufficient to generate sufficient surrogates to use the statistical method described earlier.

Survey of Literature Results

Nonbursting Dopamine Neurons

To determine whether dopamine neurons were intrinsically capable of generating chaotic firing patterns, Lovejoy et al. 2001 examined ISI data from substantia nigra pars compacta dopamine neurons both in a slice preparation treated with the small conductance (SK) potassium channel blocker apamin and from irregularly firing neurons recorded extracellularly in vivo in anesthetized rats. The hypothesis underlying this work was that the irregular firing pattern observed in vivo results from the modulation of the SK current in the intact animal, and this firing pattern could best be mimicked in vitro by reducing the level of SK conductance (see Fig. 1). Note that the distributions of the ISIs in vitro in the presence of 300 nM apamin (Fig. 1b1) and in irregularly firing neurons recorded in vivo (Fig. 1b2) are reasonably similar. Since the focus was on irregular firing and not on bursting, only records that contained fewer than six bursts of three or more spikes (burst defined as in Grace and Bunney 1984b) in a total of 1,000 ISIs and fewer than 2% doublets were examined. Lovejoy et al. (2001) generated a time series from the ISI data as already described and made iterative forecasts by predicting the time course starting from each observation in the half of the data set not used to generate the attractor for as far into the future as the data allowed. The prediction error increased exponentially in time as expected for a chaotic time series. However, these results were not compared with surrogate data generated from the original ISI series. Canavier et al. (2004) made such a comparison using surrogates created from randomly shuffled ISIs, which destroy all temporal structure, not just the dynamic nonlinear structure as in the Gaussian-scaled surrogates described earlier. We found that the prediction error did indeed scale exponentially in time for the original data, as evidenced by an initial straight line on a semi-log plot of prediction error for both the in vitro (Fig. 3a, *open squares*) and in vivo data (Fig 3b, *open squares*). The prediction error was quantified in bits (Wales 1991) as $\log_2[1-p]$ bits, where p is the Pearson's correlation coefficient between the predicted and actual SDF. However, so did the prediction error for the randomly shuffled intervals in which all temporal sequence structure was removed. Therefore, the apparent exponential scaling was an artifact

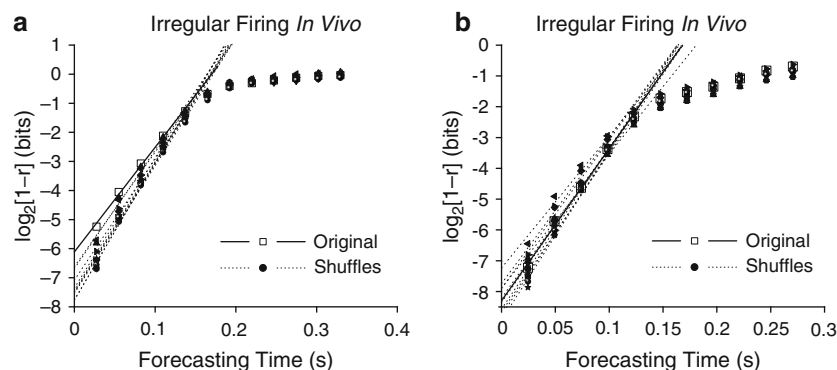


Fig. 3 Application of nonlinear forecasting to SDF generated from ISIs. The rate of loss of predictability in bits is given on the y-axis as a function of the prediction time for both the original data and randomly shuffled surrogates. A straight line on a semilog plot indicates exponential decay of predictability. Although exponential decay of predictability is a hallmark of chaos, in this case it is spurious because the dotted lines were produced from randomly shuffled ISI series with no temporal structure. (a) Predictions from original and shuffles from neurons in vitro treated with apamin. (b) Predictions from neurons in vivo. Adapted from Canavier et al. (2004)

of the creation of the SDF using a Gaussian window, which introduced spurious predictability into the time series, combined with the ISI distributions of the irregularly firing dopamine neurons (Fig. 1b). The distribution of the ISIs apparently limits the falloff in predictability compared with that of Poisson-distributed intervals, which do not produce SDFs that exhibit exponential falloff (Lovejoy et al. 2001). The SDF method was robustly able to detect chaos (Canavier et al. 2004) in a model known to be in a chaotic regime, and therefore, its inability to detect chaos in the experimental data may simply mean that the data are too noisy or that ISIs do not allow for adequate sampling of the system dynamics.

All other studies of nonlinear forecasting in dopamine neurons forecast directly from the ISI series without creating the SDF and forecast only one ISI into the future. Since the SDF method did not give conclusive evidence of chaotic structure, predictions using this methodology and the random Gaussian surrogates described earlier were then applied. The null hypothesis that the data were produced by a linear process with additive Gaussian noise and a static nonlinearity was rejected for four of five neurons in vitro and two of nine neurons in vivo. The Spearman rank correlation coefficients (r_s) between the predictions and the actual ISIs were 0.386 ($p < 0.0001$), 0.381 ($p < 0.005$), 0.311 ($p < 0.015$), and 0.255 ($p < 0.0003$) for the in vitro records that differed significantly from the Gaussian-scaled surrogate, and 0.13 ($p < 0.005$) and 0.16 ($p < 0.0001$) for the in vivo records. The use of time-reversed records produced mixed results, producing correlation coefficients similar to the original data for the in vitro records, but values in the range of the surrogates for the in vivo records. Overall, the evidence from this study for nonlinear sequence-dependent structure was not compelling.

Bursting Dopamine Neurons: Basal Nonlinear Sequence Dependence and the Effect of Forebrain Hemisection

Hoffman et al. (1995) recorded ISIs from substantia nigra pars compacta dopamine neurons in unanesthetized rats in low *cerveau isole* preparations to detect basal nonlinear sequence dependent predictability. The basic questions addressed by this study and the subsequent one described in the following section were as follows. Does the nonlinear structure of DA neuron ISIs arise primarily from the burst-associated properties of the neuron itself or from the temporal organization of its synaptic inputs? In contrast to Canavier et al. (2004) which excluded neurons that exhibited too many bursts, Hoffman et al. (1995), excluded data with too few bursts, as the records examined were required to have more than ten bursts in 2,100 or 2,400 ISIs. They obtained a r_s of 0.25 ± 0.09 for 15 cells, and ten differed significantly from their Gaussian-scaled surrogates ($p < 0.0005$). However, the specific cells identified as being significantly different were not entirely consistent when results from embedding dimensions of 4 and 7 were compared. The correlation dimension identified the dimension of 9 of 15 cells as being significantly different from their controls, but there was no correlation between the z-scores obtained for nonlinear forecasting and correlational dimension, which casts doubt on the correlational results because forecasting is the more robust measure (Yunfan et al. 1998). The authors referred to the correlation dimension as correlational complexity instead of dimension, and noted that in their study, this quantity was highly sensitive to embedding dimension. This sensitivity also decreases confidence in the meaning of this measure (see *Correlation Dimension*) in the context of

nonlinear dynamics. Bursting did not differ significantly between the surrogates and the actual data, which indicates that bursting alone is not the primary source of nonlinear structure in the data.

Hoffman et al. (2001) followed up with a study of anesthetized rats with and without basal forebrain hemisection. The underlying hypothesis was that the decrease in the coefficient of variation (CV) observed in dopamine neurons in a slice preparation compared with that in an intact animal (Shepard and Bunney 1988) results at least partially from network interactions with the forebrain. The hemisection caused a significant reduction in CV as well as a significant reduction in predictability, although the former did not account for the latter statistically. The mean r_s for the unlesioned animals was 0.313 ± 0.076 , which is comparable to the results obtained in their original study, whereas the mean for the hemisected animals was 0.184 ± 0.105 ($p < 0.002$). Gaussian surrogates were not used to determine whether the decrease in predictability derived primarily from linear or nonlinear sources, but based on the reported values for control and surrogates in other studies (Di Mascio et al. 1999a, 1999b), it is likely that the degradation in predictability is due to the loss of nonlinear structure. Again, no correlation between burst firing and predictability was found.

Effects of Aging and of Serotonin Denervation

Di Mascio et al. (1999a) applied analytical methods identical to those of Hoffman et al. (1995) to sequences of 2,000 ISIs recorded from ventral tegmental dopamine neurons in anesthetized rats. They compared young, adult, and aged rats. The mean r_s for the 12 records from young rats was 0.22 ± 0.10 , which was significantly different from the mean of 0.11 ± 0.08 obtained for their Gaussian-scaled surrogates ($p < 0.0001$). For the 20 cells from adult rats, the mean r_s was 0.18 ± 0.09 , which was also significantly different from the mean of 0.11 ± 0.06 observed for the surrogates ($p < 0.0001$). On the other hand, the mean r_s of the records from the aged rats was only 0.11 ± 0.09 , which was comparable to that of the Gaussian-scaled surrogate data, which was 0.09 ± 0.05 ($p < 0.18$). Therefore, the young and adult rats showed strong evidence of nonlinear sequence structure that was lacking in the aged rats. No consistent, significant differences were found in measures of bursting activity across the three groups, and hence, differences in bursting could not account for differences in predictability.

Di Mascio et al. (1999b) also investigated, using similar methods, the effects of serotonin denervation mediated by whole brain 5,7-dihydroxytryptamine (5,7-DHT) lesions on ventral tegmental area dopamine neurons in anesthetized rats. Twelve control records had an r_s of 0.26 ± 0.08 , whereas

that of the Gaussian-scaled surrogates was 0.16 ± 0.07 ($p < 0.00003$). The 5,7-DHT lesioned group had an r_s of 0.19 ± 0.09 , which did not differ significantly from their Gaussian-scaled surrogates 0.16 ± 0.05 ($p < 0.15$). This group also found no significant correlation between bursting and predictability.

Conclusions

The determination of nonlinear structure in noisy ISI data is nontrivial and fraught with pitfalls. Nonetheless, the judicious use of controls such as Gaussian surrogate data increases confidence that actual nonlinear sequence structure is being measured. The decrease in predictability observed after forebrain hemisection (Hoffman et al. 2001) or serotonin denervation (Di Mascio et al. 1999b) supports the hypothesis that network interactions rather than intrinsic dynamics are largely responsible for nonlinear sequence-dependent predictability in midbrain dopamine neurons. Somewhat surprisingly, no evidence has been found linking the prevalence of bursting to the amount of nonlinear structure, despite the widespread agreement that bursting is a salient network phenomenon. Furthermore, in contrast to the idea that chaotic complexity is detrimental to system performance, the decrease in nonlinear sequence structure with age (Di Mascio et al. 1999a), serotonin denervation (Di Mascio et al. 1999b), and forebrain hemisection (Hoffman et al. 2001) supports the idea that complexity in the sequence structures of ISI series in midbrain dopamine neurons enhances rather than degrades system functionality, as aged, lesioned, and hemisected animals are likely to be less functional than their non-aged or intact counterparts.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 10

Age-Dependent Changes in Dopaminergic Neuron Firing Patterns in Substantia Nigra Pars Compacta

Yoshiyuki Ishida, Tatsuya Kozaki, Yoshikazu Isomura, Sachiko Ito, and Ken-ichi Isobe

Abstract Dopaminergic neurons in the substantia nigra pars compacta modulate complex motor control. Nigral dopaminergic neurons exhibit three different firing patterns in vivo: a pacemaker mode, a random mode, and a burst mode. These firing patterns are closely related to motor control. However, the changes in the proportion of the firing patterns with respect to age have not been fully established. To clarify the age-dependent changes in the proportion of dopaminergic firing patterns, we used single unit extracellular recordings in male F344/N rats. We observed that, with age, the distribution of the spikes fired by dopaminergic neurons shifts from pacemaker to random mode, and then from random to burst mode. These results suggest that the age-dependent changes in the proportion of nigral dopaminergic firing patterns may have an effect on motor function.

Keywords Aging • Electrophysiology • F344 rat • Firing pattern • Substantia nigra pars compacta

Abbreviations

CV Coefficient of variation
DA Dopamine
ECG Electrocardiogram
EEG Electroencephalogram

SNC Substantia nigra pars compacta
Str Neostriatum

Introduction

The activity of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNc) is closely related to complex motor control (Payne et al. 2000). Nigral dopaminergic neurons exhibit three firing patterns in vivo: a regular, pacemaker-like mode, a random mode, and a burst mode (Wilson et al. 1977; Grace and Bunney 1984; Tepper et al. 1995). Functionally, the electrical stimulation of the medial forebrain bundle with trains of stimuli resembling burst firing of DA neurons leads to a greater increase in the extracellular dopamine levels in target regions than with regularly spaced stimuli having the same average frequency of DA neurons (Gonon 1988). On the other hand, the application of a DA agonist causes animals to change their behavioral responses (Campbell et al. 1984). Thus, these findings allow us to speculate on the correlation between the activity of DA neurons in SNc and behavioral responses to environmental stimuli in animals, such as exploratory behavior (Freeman et al. 1985), operant tasks (Hyland et al. 2002), and reward-mediated learning processes (Schultz et al. 1997).

Although these behaviors seem to decline during the normal aging process, little is known about the change in the activity of DA neurons in SNc during aging. For example, the activity of DA neurons affects the plasticity of their axon terminals (Bilbao et al. 2006), and our previous data have shown that with age, DA neurons might stimulate axonal branching in their target area such as the neostriatum (Ishida et al. 2007). Thus, this suggests that the activity of DA neurons changes during aging.

In this study, we focused on the age-dependent changes in the distribution of DA neuron firing patterns in SNc, using in vivo electrophysiological techniques to investigate the activity of DA neurons of rats in different age groups.

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Material and Methods

Animals

Male F344/N rats (four groups; 6, 12, 18 and 24 months of age, $N=5$ for each age group) were used. Animals were housed with food and water available ad libitum on a 12-h light/dark cycle. All animal procedures complied with the Animal Research Facilities Committee of the National Institute for Longevity Sciences.

Electrophysiology

Animals were anesthetized with urethane (1.2 g/kg, i.p.), and lidocaine was applied locally to all incisions. Rectal temperature was maintained at 36.5°C by a heating pad, and ECG and EEG were monitored continuously during the experiments. Stimulating electrodes of the bipolar type consisted of two insulated stainless steel wires (diameter 200 μ m, tip separation 0.5 mm). Electrical pulses for the stimulus site were 0.5 ms in duration with currents ranging from 0.1 to 5.0 mA, and the cycle of stimulation was 1.5 s. The electrodes were stereotactically guided into the neostriatum (Str) (A: 1.0 from bregma, L: 3.7, D: 4.0). The electrical activity of SNc neurons was extracellularly recorded with glass pipette microelectrodes filled with 2 M NaCl. Electrode resistance ranged from 10 to 20 M. A recording electrode was inserted from a point (A: 2.1 from lambda, L: 2.0), and SNc neurons were usually encountered 6.8 - 8.0 mm below the cortical surface. To avoid sampling bias, 16 - 26 SNc neurons were recorded in each animal by moving a recording electrode rostrocaudally or mediolaterally within a distance of

about 200 μ m. The DA neurons in the SNc were identified according to well-established criteria (Deniau et al. 1978; Guyenet and Aghajanian 1978). Briefly, nigral dopaminergic neurons exhibited a characteristic wide spike duration (until 2 ms), and slow spontaneous firing between 0.5 and 6 Hz. SNc neurons exhibited responses from striatal stimulation and were considered to be antidromic in nature if the following criteria were satisfied: (1) constant latency of the initial segment spike at a low stimulation frequency (1 Hz), (2) ability to follow stimulation at high frequencies (>200 Hz), and (3) collision with spontaneous or orthodromic spikes. The stimulating current was adjusted to a value that was just sufficient to elicit an antidromic response to every stimulus. The threshold current was measured by varying the stimulating current in 0.01 mA steps.

Data Analysis

The spikes of data were collected and analyzed using Chart software (ADI USA). At least 250 spikes of baseline data were collected from each neuron. Interspike interval histograms or autocorrelograms were constructed using a bin width of 4 ms for intervals up to 1 or 2 s and were used to qualitatively classify neurons as firing in pacemaker, random, or burst-firing patterns (Fig. 1). Regularly occurring peaks in the autocorrelogram were characteristic of the pacemaker firing pattern. An initial trough that rose smoothly to a steady state was classified as the random firing pattern. Bursts were defined as beginning with a pair of spikes with an interspike interval of 80 ms or less and terminating with a pair of spikes with an interspike interval of 160 ms or greater (Grace and Bunney 1984). The regularity of the

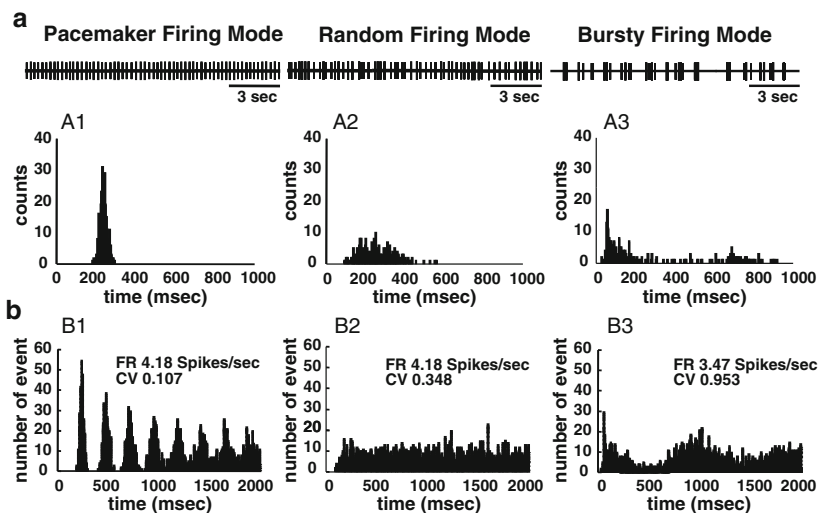


Fig. 1 (a) Interspike interval histograms (*upper panels*) and (b) autocorrelograms (*lower panels*) of representative DA neurons in the SNc exhibiting three firing patterns: pacemaker firing at 6 months (**a1** or **b1**), random firing at 24 months (**a2** or **b2**), and burst firing at 24 months (**a3** or **b3**). The first several seconds of the spike train used to create both the interspike interval histogram and the autocorrelogram is shown above each respective interspike interval histogram

firing patterns was indexed using the coefficient of variation (CV), calculated as the standard deviation of the interspike interval divided by the mean interspike interval. The data were expressed as means $\pm$ SE, and statistical tests used for comparing the different types of firing patterns were chi-square tests with Tukey's method and one-way analysis of variance with a Bonferroni/Dunn post hoc analysis.

Results

The total numbers of DA neurons recorded from five animals in each age group were as follows: 6 months of age, $n=46$; 12 months, $n=50$; 18 months, $n=50$; 24 months, $n=56$. These neurons were antidromically activated from the ipsilateral neostriatum with characteristics that identified them conclusively as dopaminergic nigrostriatal neurons. To quantify a change in the proportion of DA neuron firing patterns with age, we first focused on the percentage of cells exhibiting each of the firing patterns at different ages (Fig. 2a). The percentage of neurons exhibiting the pacemaker mode decreased considerably between 12 and 18 months of age, whereas the percentage of neurons with the burst mode increased gradually with age. The percentage of neurons exhibiting the random mode greatly increased between 12 and 18 months of age and decreased between 18 and 24 months of age. Figure 2b shows that the percentage of neurons exhibiting the pacemaker mode slightly increased between 6 (39.1%) and 12 months of age (50.0%), then the percentage significantly decreased between 12 and 18 months of age (14.0%) and was maintained between 18 and 24 months of age (21.4%). In addition, the percentage of

random firing neurons slightly decreased between 6 (54.3%) and 12 months of age (46.0%), significantly increased between 12 and 18 months of age (72.0%), and then significantly decreased between 18 and 24 months of age (46.4%). The percentage of burst firing neurons was maintained between 6 (6.5%) and 12 months of age (4.0%), and then gradually increased between 12 and 18 months of age (14.0%). The increase in the percentage of burst firing neurons at 24 months (32.1%) was significantly higher than that at 6, 12, or 18 months.

The CV is thought to be a good measure of burst firing or bursting state. Figure 3a shows that the increase in the CV of burst firing neurons at 24 months (0.571 ± 0.040) was significantly higher than that at 6 (0.404 ± 0.047), 12 (0.340 ± 0.023), or 18 months (0.423 ± 0.027).

Figure 3b shows the mean firing rate of DA neurons among each age group. The mean firing rate slightly increased between 6 (3.623 ± 0.199 spikes/s) and 12 months (3.999 ± 0.225 spikes/s) and then maintained between 12 and 18 months (4.033 ± 0.224 spikes/s). Then the rate decreased between 18 and 24 months (3.207 ± 0.180 spikes/s).

Discussion

In this study, we showed age-dependent changes in the proportion of DA neuron firing patterns. We observed that the percentage of neurons exhibiting the pacemaker mode decreased between 12 and 18 months, whereas that for the random mode increased between 12 and 18 months and then decreased between 18 and 24 months. We also found that the percentage of neurons exhibiting the burst mode significantly

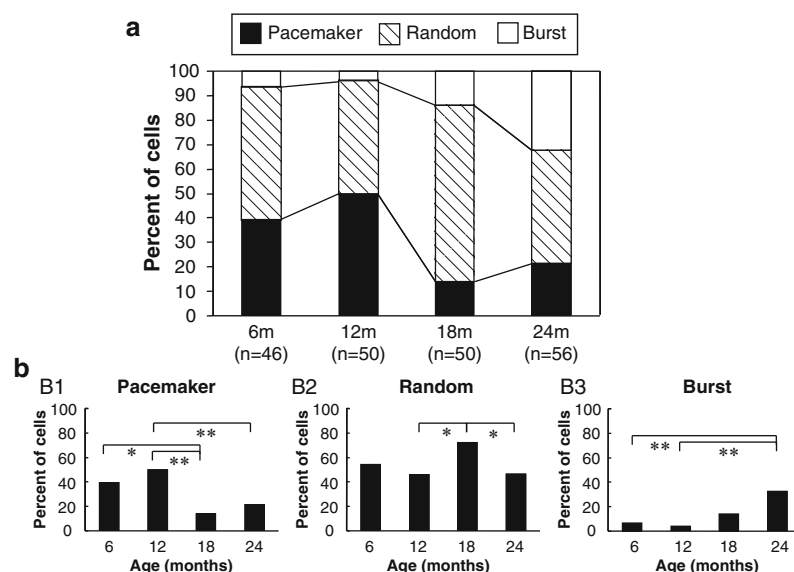


Fig. 2 Age-dependent changes in the proportion of DA neuron firing patterns. (a) The overall percentage of spikes fired in each firing pattern. (b) Chi-square tests with Tukey's method were used to test the patterns of pacemaker (b1), Random (b2) and Burst (b3). * $P < 0.05$; ** $P < 0.01$

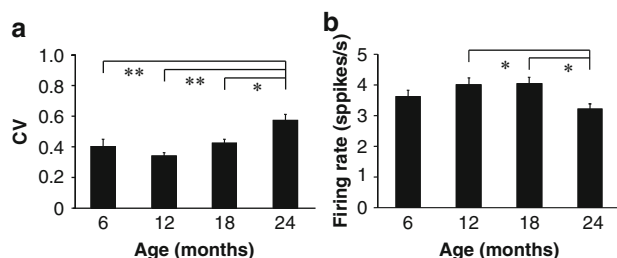


Fig. 3 The age-dependent changes in the mean CV and firing rate of DA neurons. (a) Age-dependent changes in the CV of DA neurons. $F(3,198)=8.079$, $P<0.0001$. (b) Age-dependent changes in the firing rate of DA neurons. $F(3,198)=7.962$, $P=0.013$. One-way analysis of variance with a Bonferroni/Dunn post hoc analysis was conducted. * $P<0.05$; ** $P<0.01$

increased between 18 and 24 months of age. These results suggest that, with aging, there is a decreased proportion of pacemaker firing patterns in the DA neurons and an increased proportion of random or burst firing patterns.

We have shown that there is a decrease in the proportion of pacemaker mode in the DA neurons and an increase for that of the bursting pattern. The bursting stimulations of the dopaminergic pathway were twice as potent as regularly spaced ones for enhancing the extracellular DA concentration (Gonon 1988). Also, the high extracellular overflow evoked by a burst is due to an accumulation of the released DA, whereas the extracellular DA was readily eliminated between every action potential at lower frequencies (Chergui et al. 1994). Also, the activity of DA transporters decreased significantly between young (6 and 12 months) and aged (18 and 24 months) F344 rats (Hebert and Gerhardt, 1998). In addition, a decrease in DA receptors in the Str of aged F344 rats has been observed (Sakata et al. 1992; Suzuki et al. 2001). These results suggest that with age, the increased proportion of burst firing patterns of DA neurons facilitate DA release and the accumulation of the released DA enhances the sensitivity of the remaining DA transporters and DA receptors.

We found a significant increase in the mean firing rate of DA neurons between 12 and 24 months and between 18 and 24 months. This is important because the plasticity of axon terminals of DA neurons is affected by the activity of DA neurons (Bilbao et al. 2006). Previously, we reported age-dependent changes in the dopaminergic innervation of the neostriatum of F344 rats that suggested an increase in axon terminal density of Str between 18 and 24 months and significantly increased axonal branching in Str between 6 and 12 months and 6 and 24 months (Ishida et al. 2007). Thus, these results suggest that the age-dependent change in the activity of DA neurons might be involved in DA axon terminal branching with age.

Our finding is inconsistent with the report by Freeman et al. (1989), who concluded that the proportion of neurons

with burst firing pattern did not change between 3 and 24–28 months in F344 rats anesthetized with chloral hydrate. This discrepancy might be partly solved in the future by conducting studies with increased sample size, because the proportion of neurons with the burst firing pattern at 24–28 months (56%; 24/43) tended to be higher than that at 3 months (40%; 10/25). In addition, the proportion of neurons with the firing pattern in rats with chloral hydrate is higher than that with urethane. In other respects, our data are in good concordance with their findings of no significant differences in other electrophysiological parameters, such as firing rate. Thus, we confirm that the proportion of neurons with burst firing patterns increases with age.

It would be useful to further investigate whether these changes with age are due to the changes with age in the channels expressed on DA neurons or in their input (neostriatum, substantia nigra pars reticulata and globus pallidus).

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 11

The Neurobiology of the Substantia Nigra Pars Compacta: from Motor to Sleep Regulation

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Abstract Clinical characteristics of Parkinson's disease (PD) are the result of the degeneration of the neurons of the substantia nigra pars compacta (SNpc). Several mechanisms are implicated in the degeneration of nigrostriatal neurons such as oxidative stress, mitochondrial dysfunction, protein misfolding, disturbances of dopamine (DA) metabolism and transport, neuroinflammation, and necrosis/apoptosis. The literature widely explores the neurotoxic models elicited by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA). Because of the models, it is known that basal ganglia, particularly substantia nigra, have been related to a diversity of functions, from motor to sleep regulation. Nevertheless, a current debate concerning the role of DA on the sleep–wake cycle is in progress. In summary, it is suggested that the dopaminergic system is implicated in the physiology of sleep, with particular regard to the influence of the SNpc neurons. The understanding of the functioning and connectivity of the SNpc neurons has become fundamental to discovering the neurobiology of these neurons.

Keywords 6-OHDA • Motor • MPTP • Sleep • Substantia nigra pars compacta

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease afflicting about 1% of people over 65 years old and 4–5% of people over 85% (Lang and Lozano

1998). Major clinical features at presentation include the asymmetric onset of bradykinesia, rigidity, rest tremor, and disturbances in balance. These are the result of the degeneration of the neurons of the substantia nigra pars compacta (SNpc), which leads to the subsequent reduction of dopaminergic input to the striatum. Moreover, there is a degeneration of neurons of selected brain stem nuclei (locus coeruleus, raphe nuclei, dorsal motor nucleus of the vagus), cortical neurons (particularly within the cingulate gyrus and the entorhinal cortex), the nucleus basalis of Meynert, and pre-ganglionic sympathetic and parasympathetic neurons (Pulst 2003). These apparently nonmotor features progress and come to dominate the later onset of PD, producing symptoms that include cognitive decline, depression, gastrointestinal and genitourinary disturbances, and sleep abnormalities (Schapira et al. 2006). Nevertheless, the dopamine (DA)-containing neurons seem to be the key players in PD.

Several mechanisms are implicated in the degeneration of nigrostriatal neurons such as oxidative stress, mitochondrial dysfunction, protein misfolding, and disturbances of intracellular calcium and iron homeostasis, besides polymorphisms in genes regulating DA metabolism and transport, neuroinflammation, and necrosis/apoptosis (Dauer and Przedborski 2003; Teismann et al. 2003a; Barnham et al. 2004). This profusion of events suggests the existence of numerous possible targets for the development of therapeutic strategies. The three main considered developments that have led to progress in the medical management of PD have focused on improvements in dopaminergic therapies, (including those aimed at managing or preventing the onset of motor complications), the identification of nondopaminergic drugs for symptomatic improvements, and the discovery of compounds to modify the course of the disease (Schapira et al. 2006).

Sleep disturbances are frequent symptoms that have recently been recognized in PD. The disturbances include insomnia and abnormal movements during sleep (e.g., periodic leg movements, rapid eye movement (REM) sleep behavior disorders, and excessive daytime sleepiness). Insomnia has a serious impact on a patient's quality of life.

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REM sleep behavior disorders are enacted dreams that can cause patient or bed-partner injuries. Daytime sleepiness exposes the patients to risk of a driving accident. The mechanisms of these sleep disturbances are as yet to be fully understood, but they include a clear dopaminergic component. In this sense, studies of experimental animal models of PD constitute fundamental tools that provide important light on the neurobiology of the dopaminergic neurons. In fact, the discovery that a simple molecule (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) was capable of inducing virtually all of the motor (and more recently sleep) signs of PD led to a resurgence of research in PD (Pulst 2003; Lima et al. 2007).

The objective of this review is to contextualize the physiological participation of dopaminergic neurons (within the SNpc) in motor behavior and sleep regulation. The majority of the data regarding these issues were accomplished with the support of animal models of PD. Finally, one point must be stressed at the outset of this review paper. Studies regarding other experimental animal models that do not include the neurotoxin MPTP or 6-hydroxydopamine (6-OHDA) were briefly mentioned within the text. This should not be taken as evidence undermining the significance of the models (e.g., rotenone, reserpine, genetic models, etc.), but merely as a deliberate choice made by these authors.

Dopamine in Perspective

Dopamine was first synthesized in 1910, but it was not until the mid-1950s that it began to emerge as a substance of importance in its own right rather than just an intermediary in the formation of noradrenalin (Marsden 2006). Although the 1960s and 1970s were mainly concerned with establishing an independent role for DA and making the first tentative steps in determining its physiological and behavioral functions. The 1980s were the receptor years with the development of techniques allowing the study of links between specific receptors and the function of specific DA pathways within an increasingly complex system of neuronal interactions (Marsden 2006). The first evidence for the presence of a dopaminergic receptor in the brain came from experiments showing the stimulation of adenylyl cyclase activity by DA (Kebabian et al. 1972). Later, Seeman began to search for the antipsychotic receptor and identified a site that could be labeled by both DA and haloperidol; the binding of both was inhibited by nanomolar concentrations of unlabeled haloperidol. This site was originally called the 'neuroleptic/DA receptor' (Seeman et al. 1976), but was later renamed the DA D₂ receptor in a breakthrough paper that classified the DA receptors into D₁ and D₂, based on their pharmacology and coupling to adenylyl cyclase (Kebabian and Calne

1979). Such discoveries occurred in parallel with the finding of the participation of brain DA and noradrenalin systems in aggressive behavior in rats that had been REM sleep deprived (Carlini et al. 1977). Complementary studies concerning this DA participation indicated, through behavioral and pharmacological detection, a massive motor exacerbation elicited by REM sleep deprivation, originally described as a result of dopaminergic supersensitivity (Tufik et al. 1978; Tufik 1981a, b). Afterward, the autoradiographic analysis of D₁ and D₂ receptors under the REM sleep deprivation paradigm proved the previous reports biochemically (Nunes et al. 1994). Those findings began the first embryo of the idea suggesting a possible role for DA on sleep regulation (Carlini 1983).

The discovery of DA and the description of an analytical method for its analyses were followed very quickly by reports of markedly depleted levels of DA in the basal ganglia of individuals dying from PD (Iversen and Iversen 2007). Ehringer and Hornykiewicz in Vienna were the first to report such findings in 1960, and Birkmayer and Hornykiewicz published the first positive findings with 3,4-dihydroxyphenylalanine (L-DOPA) treatment 1 year later (Ehringer and Hornykiewicz 1960; Birkmayer and Hornykiewicz 1961). Similar results were reported almost simultaneously in Canada (Barbeau and al. 1961). However, it took several more years before Cotzias in the USA developed the first practical dosage regime for the routine use of L-DOPA in the treatment of PD – still one of the major achievements in the rational development of novel therapies for neurological diseases (Cotzias 1968). L-DOPA therapy has been further improved by its combination with carbidopa or benserazide to prevent metabolism of L-DOPA in the liver, by the development of slow release formulations allowing more 'on' time and fewer 'off' periods, and more recently by the advent of various DA receptor agonists as supplements to L-DOPA or as monotherapy in the early stages of the illness (Thomson et al. 2001).

Animal Models of Parkinson's Disease

The lack of knowledge about PD etiology and pathogenesis drove the researchers toward the animal models. An animal model is defined as an animal that has disease or injury similar to that of humans. Ideally, it should have a similar etiology and function to the human equivalent, and it is necessary that many aspects of the disease are replicated, which in turn hinder the development of new therapeutic approaches (Lane and Dunnett 2007). Although recent genetic discoveries have led to a number of different genetic models of PD, none of these shows the typical degeneration of dopaminergic neurons. Thus far, among the various accepted

Table 1. Comparative board of PD models in terms of behavioral, molecular and sleep alterations

Model	Progressive loss of dopaminergic neurons	Motor deficits	TH expression reduction	COX-2 induction	Sleep disruption	References
Single MPTP intranigral injection	–	±	+	+	+	Da Cunha et al. (2001); Miyoshi et al. (2002); Perry et al. (2004); Braga et al. (2005); Ferro et al. (2005); Lima et al. (2006); Lima et al. (2007); Reksidler et al. (2007); Capitelli et al. (2008)
Intranasal MPTP administration	?	+	+	?	?	Prediger et al. (2006); Franco et al. (2007)
Repeated MPTP intranigral injection	–	+	+	+	?	Reksidler et al. (2008)
MPTP mouse model (i.p. injection)	?	+	+	+	?	Heikkilä et al. (1984); Sundström et al. (1987); Gainetdinov et al. (1997); Tatton and Kish (1997); Sedelis et al. (2001); Serra et al. (2002); Teismann et al. (2003); Laloux et al. (2008); Meredith et al. (2008)
6-OHDA	±	+	+	+	+	Ungerstedt (1968); Ungerstedt (1971a,b); Faull and Lvery (1969); Lee et al. (1996); Deumens et al. (2002); Lima et al. (2006)
LPS	–	–	+	+	?	Lima et al. (2006)

experimental models, neurotoxins have remained the most popular tools to produce selective neuronal death in both in vitro and in vivo systems (Bové et al. 2005). The literature widely explores the key neurotoxic models elicited by MPTP, 6-OHDA, and rotenone. Each substance produces characteristics that partially recapitulate the disease, although molecular, neurochemical, cellular, electrophysiological, and behavioral alterations are particularly different (Table 1).

MPTP Model

The MPTP model has served us extraordinarily well in terms of elucidating the function and circuitry of the basal ganglia in the presence of a nigrostriatal deficit, and has been widely used to test new treatments for the disease. Nevertheless, MPTP does not typically manifest all of the pathologic features of the disease, in particular Lewy body-like inclusions or sustained motor impairment. The history that permeates this neurotoxin starts back to the early 1980s when a number of young adults were seen at several California emergency rooms with bizarre symptoms, including rigidity, bradykinesia, some tremor, and postural instability, which responded to L-DOPA treatment, as with PD (Langston et al. 1983; Langston and Ballard 1983). The appearance of this Parkinsonian syndrome at this age was surprising in that PD usually manifests itself generally between 50 and 60 years of age. It was eventually discovered that these patients were addicted to homemade heroin. Analysis of the residual drug confirmed the presence of MPTP (Langston et al. 1983).

The dopaminergic neurodegeneration that occurs following the administration of MPTP in animals has provided a useful model of Parkinsonism, because it presents a high face and predictive validity, since it induces pathologies and pharmacological responses similar to those seen in humans. MPTP is highly lipophilic and readily crosses the blood–brain barrier. Monoamine oxidase-B (MAO-B) in glial cells converts MPTP to 1-methyl-4-phenylpyridinium (MPP⁺) (Chiba et al. 1984), which is taken up via the neuronal DA transporter and accumulates in dopaminergic neurons. Mice that lack this transporter are protected from MPTP toxicity (Gainetdinov et al. 1997). MPTP and MPP⁺ cause massive DA release, which is then metabolized by oxidative degradative processes, including MAO-B, forming reactive oxygen species, such as hydrogen peroxide (H₂O₂) and superoxide radicals (O₂^{•−}), which themselves can be toxic (Speciale 2002). Reactive oxygen species are produced normally by DA metabolism and exert important physiological functions, including their participation in the defense against the intrusion of foreign bodies. However, the accumulation of these

oxygen species may damage cells by the oxidative degradation of critical molecules such DNA, lipids, and proteins (Cui et al. 2004). Numerous neurochemistry studies have shown that MPTP administration predominately damages the nigrostriatal pathway, causing cell loss in the SNpc (Heikkilä et al. 1984; Sundström et al. 1987; Gevaerd et al. 2001) and DA depletion in the neostriatum (Sonsalla and Heikkilä 1986; Da Cunha et al. 2001; Perry et al. 2004). Such effects observed in the animal model mimic the neurochemical and histological alterations observed in PD (Meredith et al. 2008). In most of these studies, a decrease in locomotion and/or rearing was observed following MPTP administration (Sedelis et al. 2001; Lima et al. 2006; Prediger et al. 2006; Reksidler et al. 2008).

It is well established that MPTP produces, in both humans and monkeys, an irreversible and severe Parkinsonism, but the same features are not completely recapitulated when rodents are used. In a study with Swiss mice subjected to systemic MPTP 30 mg kg⁻¹ per day for 5 consecutive days (Tatton and Kish 1997), death of nigral neurons occurred in apoptotic form on day 6 after MPTP discontinuance was demonstrated, and striatal DA was depleted by about 59% 1 day after MPTP discontinuance, with a stable trend to recovery (DA depletion of about 28%) from day 3 to day 14 after MPTP (Serra et al. 2002). We have shown in previous studies that the administration of MPTP directly into the SNpc of rats causes a partial loss of dopaminergic neurons, depletion of striatal DA, and upregulation of the proinflammatory enzyme cyclooxygenase-2 (COX-2) resulting in sensorial, memory, and motor deficits with temporary impairment, modeling the early phase of PD (Da Cunha et al. 2001; Miyoshi et al. 2002; Perry et al. 2004; Braga et al. 2005; Ferro et al. 2005; Lima et al. 2006; Reksidler et al. 2007). Recently, a different protocol of MPTP-induced nigrostriatal lesion in rats was proposed, with the use of intranasal administration of MPTP (Prediger et al. 2006; Franco et al. 2007). Nevertheless, this PD model, like others, reproduces the early phase of the disease, which denies the possible existence of a synergic effect of consecutive insults to the dopaminergic neurons.

In this sense, a novel protocol of MPTP nigrostriatal lesion was proposed, which consisted of repeated neurotoxin intranigral administrations to differentiate the effects of a single lesion from the effects of repeated lesions. Because there is cumulative nigrostriatal degeneration, similar to that observed during the development of the disease, a schedule of 3 day intervals between the MPTP administrations, totaling three infusions in 9 days, was elected (Reksidler et al. 2008). Such an exposure paradigm revealed a persistent locomotor deficit produced when approximately 50% of the tyrosine hydroxylase immunoreactive (TH-ir) neurons were destroyed: a condition detected at the very first time-point (1 day after the 1st infusion). In addition, this study demon-

strated that nigral TH protein expression is considered an important target for MPTP action, since that enzyme was found to be downregulated after all the MPTP infusions. In conclusion, the authors suggest that TH expression is instrumental for locomotor behavior, rather than merely the number of TH-ir neurons within SNpc (Reksidler et al. 2008).

6-OHDA Model

The first observation of the biological effects of 6-OHDA showed that this agent was able to induce efficient and long-lasting noradrenalin depletion in sympathetic nerves to the heart (Porter et al. 1963). A few years later, Ungerstedt in a classical experiment showed that injection of 6-OHDA into the SNpc was able to cause anterograde degeneration of the nigrostriatal dopaminergic neurons, thus generating the first animal model of PD (Ungerstedt 1968). Since this discovery, 6-OHDA has been extensively used for replicating the neuronal loss observed in PD.

The neuronal damage induced by 6-OHDA is mainly due to the massive oxidative stress caused by the toxin. Being similar to DA, 6-OHDA shows high affinity for the DA transporter, which carries the toxin inside the dopaminergic neurons. Once in the neuron, 6-OHDA accumulates in the cytosol and undergoes prompt auto-oxidation, promoting a high rate of free radical formation (mostly H₂O₂). The classical injection protocol adopts the unilateral injection into the SNpc or in the medial forebrain bundle producing massive, virtually complete lesion of nigral dopaminergic cell bodies; SNpc neurons begin to die within the first 12 h of injection, and a marked lesion of striatal dopaminergic terminals, paralleled by DA depletion, is established within 2–3 days (Faull and Lavery 1969; Lee et al. 1996; Deumens et al. 2002). An overview of the most recent morphological and biochemical findings obtained with the 6-OHDA is provided in a careful review by Simola and colleagues (2007), with particular attention being focused on the newly investigated intracellular mechanisms at the striatal level (e.g., NMDA receptors, PKA, CaMKII, ERK kinases, as well as immediate early genes, GAD67 and peptides) (Simola et al. 2007).

Unilateral nigrostriatal depletion results in a unilateral motor deficit that can be evaluated in a variety of different tests. A rat with a unilateral nigrostriatal lesion exhibits postural bias to the ipsilateral side, which is transformed into strong ipsilateral turning (rotation) after activation by the injection of a stimulant drug such as amphetamine or apomorphine. Extracellular DA is increased in the intact striatum creating an imbalance in motor activation resulting in the rotational response. The animals will also rotate ipsilaterally after a variety of activating stimuli, such as stressors

(e.g., placing in a novel environment, placing on an ice cold surface, or tail pinch), although the pharmacological methods of activation are the most widely used because of their reliability and reproducibility. Drugs are often initially screened for anti-Parkinsonian potential judged by their ability to induce contralateral rotational behavior, indicating the restoration of function in the lesioned striatum, as opposed to increased dopaminergic function in the intact hemisphere. The DA precursor L-DOPA produces robust contralateral rotations as do DA agonists such as bromocriptine and apomorphine (Ungerstedt 1971a; Reavill et al. 1980). The contralateral direction of rotation suggests preferential activation of the outputs from the striatum on the lesioned side, which is thought to be dependent upon the development of postsynaptic DA receptor supersensitivity on the striatal target neurons, after their dopaminergic deafferentation. Indeed, this behavioral data were the initial basis for the dopaminergic supersensitivity hypothesis (Ungerstedt 1971b), which has been subsequently confirmed on many occasions by changes in both the sensitivity and binding in receptor ligand assays, and is supported by the fact that agonists such as apomorphine induce strong contralateral turning at very low doses that are subthreshold for any detectable activating effect in normal animals (Ungerstedt 1971a, 1971b).

Mechanisms of Dopaminergic Neuronal Death

Over the past few decades, a large core of data originating from clinical studies, autopsy materials, and in vitro and in vivo experimental models of PD has been accumulated, which led to an increased level of understanding of the pathogenesis of sporadic PD (Dauer and Przedborski 2003). Available data would argue that the mechanism of neuronal death in PD starts with an otherwise healthy dopaminergic neuron's being hit by an etiological factor, such as mutant α -synuclein. Subsequent to this initial event, it is proposed that a cascade of deleterious factors is set in motion within that neuron made not of one, but rather of multiple, factors such as free radicals, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and apoptosis, to cite only some of the most salient. Still based on this proposed scenario, all of these noxious factors will interact with each other to ultimately provoke the demise of the injured neuron (Przedborski 2005).

Findings from the MPTP model indicate that the initial cellular perturbations include the inhibition of mitochondrial respiration. Indeed, soon after the systemic administration of MPTP to mice, its active metabolite, (MPP⁺), does concen-

trate in the mitochondrial matrix, where it binds to complex I of the electron transport chain (Przedborski et al. 2004). MPP⁺ binding interrupts the flow of electrons along the mitochondrial electron transport chain, thereby leading to an increased production of reactive oxygen species (ROS), especially of O₂⁻ radical (Przedborski et al. 2004). The pathogenic significance of such a local excess of ROS production is supported by the demonstration that mitochondrial aconitase activity is reduced in the ventral midbrain of MPTP-treated mice (Liang and Patel 2004). In addition to provoking mitochondrial oxidative stress and energy crisis, MPP⁺ also interacts with the synaptic vesicles through its binding to vesicular monoamine transporter-2 (Speciale et al. 1998). In doing so, MPP⁺ translocates into synaptic vesicles where it stimulates the extrusion of synaptic DA (Rollema et al. 1988), reminiscent of the effect of methamphetamine. The resulting excess of cytosolic DA can readily undergo auto-oxidation, thus generating a huge burst of ROS, subjecting nigral neurons to an oxidative stress. Alternatively, the oxidation of cytosolic DA can also be catalyzed by enzymes such as cyclooxygenase-2 (COX-2) (Hastings 1995), which is upregulated in the remaining nigral dopaminergic neurons in both MPTP-treated mice and rats and in human postmortem samples (Teismann et al. 2003a; Lima et al. 2006). Particularly in rats, COX-2 is strongly upregulated after neurotoxic assaults provoked by intranigral MPTP, 6-OHDA or lipopolysaccharide (LPS). It has been shown that COX-2 induction produced by LPS and by the neurotoxins suggest an increase in microglial activation in the SNpc which, by itself, can be interpreted as a manifestation of damage in the dopaminergic system (Lima et al. 2006). Considering these findings, the authors suggested that LPS could be considered as a neuroinflammatory model of PD in terms of the upregulation of COX-2 a few hours after exposure. Otherwise, LPS was surprisingly unable to replicate the motor impairment, which is the strongest characteristic of this disease, in spite of the TH content depletion achieved after MPTP by LPS intranigral injection (Lima et al. 2006). Since the role of the neuroinflammatory participation in neuronal death was identified, a number of nonsteroidal antiinflammatory drugs were tested to define some possible neuroprotective effects (a careful review about this topic is provided by Esposito et al. (2007)).

Inflammation is clearly primarily a beneficial phenomenon and in the Central Nervous System it seems to be a time- and site-specific defense mechanism designed to eliminate irreversibly damaged neurons. However, at later stages, it has been shown that inflammation can develop as an uncontrolled chronic process. Promising experimental data suggest that the inhibition of the neuroinflammatory response may reduce the degeneration of dopaminergic neurons in numerous models of PD (Whitton 2007). Sodium salicylate and aspirin, both nonselective COX inhibitors, have been

observed to significantly protect striatal dopaminergic neurons from neurotoxin-induced degeneration (Sairam et al. 2003; Di Matteo et al. 2006). However, selective COX-2 inhibitors have been suggested to prevent the degeneration of dopaminergic neurons induced by MPTP (Ferber et al. 1999; Teismann and Ferger 2001; Sairam et al. 2003; Teismann et al. 2003b; Reksidler et al. 2007). In particular, the selective inhibition of COX-2 by parecoxib was shown to be effective in counteracting motor, cognitive, and TH expression deficits caused by MPTP mainly in a time course manner, suggesting that COX-2 inhibition could be related to the modulation of dopaminergic mechanisms of neuronal plasticity (Reksidler et al. 2007). Interestingly, a cohort study of patients has shown that the risk of developing PD in regular nonsteroidal antiinflammatory drug users (for cardiovascular protection) was decreased by up to 45% compared with those who take nonsteroidal antiinflammatory drugs on a nonregular basis (Chen et al. 2003), corroborating the suggested possible neuroprotective effect in PD. It appears evident that inflammatory processes are involved in the pathophysiology of PD. Therefore, neuroinflammation is defined as a process orchestrated by activated resident

microglia cells and sustained by them and other immune cells, and might be contributing to the demise of nigral DA cells, perpetuating the neurodegenerative phenomenon (Esposito et al. 2007). Conspicuously, the inflammatory stimuli and (ROS) imbricate in one activation of NF- κ B in microglial cells, oligodendrocytes, and neurons to promote the transcription of inflammatory cytokines (IL-1 β , IL-6, interferon- γ , TNF- α), apoptosis-promoting factors (p53, Bax), COX-2 and inducible NOS (iNOS). Thus, inflammatory cytokines activate nitric oxide (NO) production via NF- κ B activation or direct promotion of iNOS transcription to lead to a cytotoxic cycle. Additionally, the neurotoxicity of L-DOPA or DA quinone formation by DA auto-oxidation has recently received attention as a DA neuron-specific oxidative stress that closely links to the representative hypotheses in the pathogenesis and progress of Parkinsonism (Berman and Hastings 1999; Asanuma and Miyazaki 2006) (for more details see Fig. 1).

There are reports stating that approximately 50% of patients develop motor complications 5 years after the initiation of L-DOPA therapy. This figure increases to approximately 70% after 15 years (Miyawaki et al. 1997). The

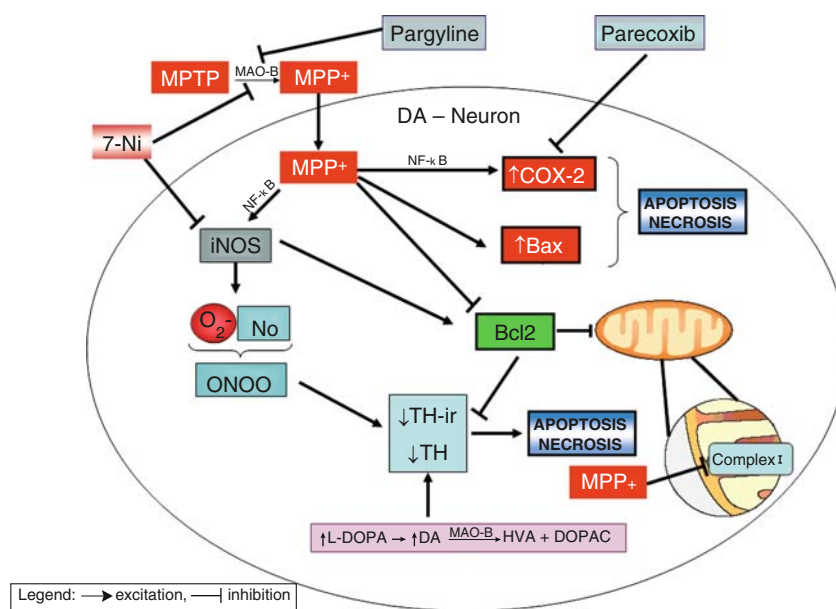


Fig. 1 Simplified schematic representation of the deleterious events leading to dopaminergic cell damage and death. Activated microglia (not represented) subsequent to immune activation or neuronal lesion caused by exposure to toxins such as MPTP/MPP⁺ or 6-OHDA can contribute to the degeneration of DA neurons by releasing neurotoxic factors such as O₂⁻, NO, ONOO⁻, and cytokines (TNF- α , IL-1 β). MPP⁺ can then activate receptor-mediated pro-apoptotic pathways within the DA neuron as well as further stimulation of the microglia in the form of iNOS and COX-2 induction. The former will lead to greatly increased NO generation and elevation of ONOO⁻, which damage the cell as a result of TH reduction and DNA damage, protein disruption and lipid peroxidation. The latter, associated with the pro-apoptotic Bax up-regulation can lead to apoptosis/necrosis of the DA neuron. Also, the metabolism of L-DOPA and DA produces quinones, semiquinones, hydrogen peroxide and other oxyradicals, which are believed to be toxic to the SN neurons. Anti-apoptotic factors such as Bcl2 counteract the pathways that lead to cell death. COX-2, MAO-B and iNOS constitute some of the most important targets for neuroprotective pharmacological interventions. Abbreviations: MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPP⁺ 1-methyl-4-phenylpyridinium, 7-Ni 7-nitroindazol, iNOS inducible nitric oxide synthase, MAO-B monoamine oxidase-B, O₂⁻ superoxide anion, NO nitric oxide, ONOO⁻ peroxynitrite anion, COX-2 cyclooxygenase-2, TH-ir tyrosine hydroxylase immunoreactive, L-DOPA 3,4-dihydroxyphenylalanine, DA dopamine, HVA homovanillic acid, DOPAC 3,4-dihydroxyphenylacetic acid

incidence of such motor problems reaches almost 100% in patients with a young-onset of PD (Thobois et al. 2005). A recent study by Reksidler and colleagues demonstrated that the intranigral administration of L-DOPA induced nigrostriatal dopaminergic destruction similar to that inflicted by MPTP in rats. Analyses of TH protein expression revealed that L-DOPA caused a reduction in the expression of this protein, one very similar to that caused by MPTP in the SNpc. Besides, the content of DA was reduced in the striatum after MPTP or L-DOPA, but an increased DA turnover was detected only for the MPTP group. Of note, the combined administration of MPTP and L-DOPA resulted in a relevant reduction of neurotoxicity to TH-ir neurons (Reksidler et al. 2009).

Role of the Substantia Nigra on Sleep Regulation

The neurobiology of sleep has quickly developed in the recent years, with remarkable progress in neurophysiological and molecular knowledge about its mechanisms. Several neurotransmitters and neuropeptides such as noradrenalin, acetylcholine, serotonin, hypocretin (orexin) have been experimentally mapped and properly allocated in the physiological cascade of sleep regulation (Monti 1982; Steriade et al. 1993; Porkka-Heiskanen et al. 1994; Steininger et al. 1999; Portas et al. 2000; Jones 2003; Siegel 2004; McCarley 2007; Monti and Monti 2007). However, until recently, the same progress that had been obtained for the other neurotransmitters was not achieved in studies about the function of DA in the neurobiology of sleep. An overview of the literature demonstrates that studies concerning a role played by DA in sleep regulation have become more numerous, especially after the 1990 decade (Lima et al. 2008b). Significant papers have been published recently regarding such involvement of the dopaminergic system in the regulation of sleep, particularly of REM sleep (Dzirasa et al. 2006; Lu et al. 2006; Dahan et al. 2007; De Cock et al. 2007; Lima et al. 2007; Lima et al. 2008a; Lima et al. 2008b).

Sleep disturbances and daytime sleepiness are well-known phenomena in PD and were reported in the original description by James Parkinson. Sleep disorders have a complex etiology related not only to the underlying neurodegenerative process but also to the motor and nonmotor features of PD and to dopaminergic therapy. A community-based study revealed that nearly two-thirds of PD patients reported sleep disturbances, which is significantly more common than patients with diabetes and healthy control subjects (Tandberg et al. 1998). Furthermore, about a third of PD patients rated their overall nighttime problems as

moderate to severe. Virtually, all patients with PD suffer from various sleep disruptions (Truong et al. 2008). In a prospective study in PD patients, cabergoline (a D₁ and D₂ agonist) treatment increased arousals, stage shifts, and awakenings, although quantitative electrophysiological measures of sleep were maintained, and subjective measures of sleep quality even improved (Hogl et al. 2003).

The prevention of sleep and enhancement of waking by DA reuptake inhibitors and DA receptors agonists are the basis for their use in the treatment of other conditions such as narcolepsy and somnolence associated with hypodopaminergic states (Pace-Schott and Hobson 2002). Moreover, PD patients present several disruptions of sleep, which are deteriorated by the use of dopaminergic D₂ receptor agonists (Larsen and Tandberg 2001; Arnulf et al. 2002; Arnulf 2006). In contrast, treatment with the D₂ antagonist and antipsychotic agent haloperidol attenuates hippocampal theta and gamma oscillations, which are characteristic of REM sleep (Dzirasa et al. 2006). The basal ganglia have been related to a wide variety of cognitive functions in addition to motor control. Experimental evidence has suggested a role for these nuclei in attention, time perception, learning and memory, while clinical data have contributed by pointing out the cognitive impairments in neurodegenerative disorders involving the basal ganglia, such as Huntington's disease and PD. Impaired basal ganglia function has also been correlated with other pathologies such as obsessive-compulsive disorder, attention-deficit disorder, autism, and schizophrenia (Mena-Segovia et al. 2003).

The current debate concerning the role of DA on the sleep–wake cycle could be split into two distinct lines of thought: the first, more traditional and conservative, assumes that DA is a neurotransmitter directly involved in events that promotes wakefulness; the second line, more recent, is struggling to demonstrate that DA is a substance dramatically related to sleep processes, in particular REM sleep. Indeed, some of these findings demonstrated that partial DA depletion causes disturbances of REM sleep without affecting motor functions (Dzirasa et al. 2006). Additionally, a robust increase in the firing of dopaminergic neurons of the ventral tegmental area (VTA) has been identified during REM sleep (Dahan et al. 2007). Moreover, clinical evidence demonstrated a transient restoration of motor control in PD patients during REM sleep (De Cock et al. 2007). Additionally, electrophysiological data indicated that the absence of half of the dopaminergic neurons located within the SNpc provoked a major impairment in the sleep–wake parameters. Some manifestations, as indicated by the decrease in the latency to slow wave sleep, initiated almost immediately after the neurotoxin microinjection. Accordingly, the SNpc neurons play an important role in regulating sleep patterns in rats, and disturbances in this particular neuronal population produced severe complications in all the sleep

parameters examined, especially in REM sleep (Lima et al. 2007). This result corroborates previous experiments with systemic injection of a single dose (2 mg kg^{-1}) of MPTP, which selectively suppressed REM sleep for 2.5–3.5 h in the cat. On the other hand, daily injection of 5 mg kg^{-1} of MPTP for five consecutive days induced a reduction of REM sleep that lasted 6–9 days after the last dose (Pungor et al. 1990). In contrast, the injection of $2.5\text{--}25 \text{ mg kg}^{-1}$ of MPTP induced a dose-dependent decrease of both REM sleep and slow wave sleep in the rat (Lelkes et al. 1991). We believe that the specificity of MPTP in producing REM sleep alterations relies on protocols that adopt low doses of the neurotoxin that generates neuronal death restricted to SNpc. With this perspective, consecutive administrations of MPTP could result in lesion spreading to other dopaminergic and non-dopaminergic areas, which consequently may result in alterations of REM sleep and also slow wave sleep. In addition, it is suggested that an acute insult to dopaminergic neurons might result in a dopaminergic supersensitivity that may potentially revert motor and REM sleep impairments promoted by neuronal lesion (Andrade et al. 1987; Lima et al. 2007). However, other results suggested that there is no correlation between the dopaminergic neuronal loss and REM sleep alteration in MPTP-treated mice (Laloux et al. 2008). This evidence postulated the existence of a biphasic time effect of MPTP on sleep parameters. In addition, Laloux et al. (2008) discussed that the existence of variations between the different protocols tested may explain the discrepancies observed in various studies of the MPTP model. Certainly, we cannot deny the participation and the plasticity of nondopaminergic structures that contribute to the generation of sleep states.

Another study contemplated the possible ability of dopaminergic D_2 blockage to produce reduction or even suppression of REM sleep, after a period of REM sleep deprivation. In this context, the findings indicated that this hypothesis is plausible according to the D_2 antagonism (promoted by haloperidol), although, the administration of D_2 agonist (piribedil) did not show the inverse (Lima et al. 2008a). It is discussed that such evidence dovetails with a possible ceiling effect of REM induction previously promoted by the sleep deprivation. Additionally, it is possible that the agonist-induced increase of REM sleep (and of slow wave sleep) depends upon the selective activation of dopaminergic D_2 presynaptic autoreceptors located in DA neurons of the ventral tegmental area by VTA, substantia nigra pars compacta by SNpc.

In summary, it is suggested that the dopaminergic system is implicated in the physiology of sleep, in particular on the influence of the SNpc neurons. It seems that nigrostriatal and mesolimbic pathways participate in triggering and sustaining the neuronal firing characteristic of REM sleep respectively (Fig. 2). This hypothesis might predict numerous

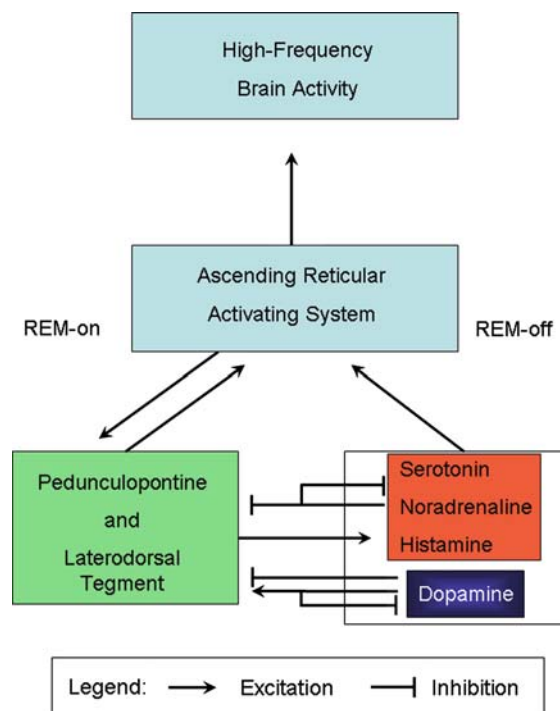


Fig. 2 Schematic model of the REM sleep generation mediated by the dopaminergic system (modified from Lima et al. 2008b). DA is represented with both excitatory and inhibitory actions projecting to REM-on neurons. Furthermore, an exacerbation of dopaminergic tone could generate an inhibitory signal to those REM-on neurons and consequently the generation of wakefulness. This modulatory characteristic is inferred as a result of the dual participation of this neurotransmitter in detriment of the respective degree of burst of its neurons present in the SNpc and VTA

clinical and therapeutic implications in different dopaminergic pathologies such as schizophrenia and PD.

Conclusion

The understanding of the functioning and connectivity of the SNpc neurons has become fundamental to reveal the neurobiology of PD. Our understanding of the role of SNpc neurons in the brain continues to unfold. The major emphasis so far has been on investigating DA in isolation from other neurotransmitter systems. In this sense, we believe that it is desirable to look at the matter more according to the classical “interconnected” Cajal’s approach, which described an extensive network of neurons through the brainstem. The various findings from animal models of PD mentioned provided compelling evidence of the different characteristics of the disease, such as motor deficit, neuroinflammation, oxidative stress, and sleep disruption. Research on dopaminergic mechanisms will play a pivotal function in the future pharmacological approach of PD, since this condition is led

by a neurodegeneration of an area well known to be responsible for motor control, which now is also being implicated in the regulation of sleep.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 12

Non-motor Function of the Midbrain Dopaminergic Neurons

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Abstract The roles of the nigrostriatal pathway are far beyond the simple control of motor functions. The tonic release of dopamine in the dorsal and ventral striatum controls the choice of proper actions toward a given environmental situation. In the striatum, a specific action is triggered by a specific stimulus associated with it. When the subject faces a novel and salient stimulus, the phasic release of dopamine allows synaptic plasticity in the cortico-striatal synapses. Neurons of different regions of cortical areas make synapses that converge to the same medium spine neurons of the striatum. The convergent associations form functional units encoding body parts, objects, locations, and symbolic representations of the subject's world. Such units emerge in the striatum in a repetitive manner, like a mosaic of broken mirrors. The phasic release of dopamine allows the association of units to encode an action of the subject directed to an object or location with the outcome of this action. Reinforced stimulus-action-outcome associations will affect future decision making when the same stimulus (object, location, idea) is presented to the subject in the future. In the absence of a minimal amount of striatal dopamine, no action is initiated as seen in Parkinson's disease subjects. The abnormal and improper association of these units leads to the initiation of unpurposeful and sometimes repetitive actions, as those observed in dyskinetic patients. The association of an excessive reinforcement of some actions, like drug consumption, leads to drug addiction. Improper associations of ideas and unpleasant outcomes may be related to traumatic

and depressive symptoms common in many diseases, including Parkinson's disease. The same can be said about the learning and memory impairments observed in demented and nondemented Parkinson's disease patients.

Keywords Addiction • Basal ganglia • Dopamine • Depression • Learning • Memory • Parkinson's disease

Abbreviations

CREB	Cyclic-AMPc response-element-binding protein
CRF	Corticotrophin-releasing factor
DSM IV	Diagnostic and statistical manual of mental disorders
GABA	Gamma amino butyric acid
GPI	Globus pallidus
HD	Huntington's disease
LTP	Long-term potentiation
NAc	Nucleus accumbens
PD	Parkinson's disease
PET	Positron emission tomography
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TH	Tyrosine hydroxylase
THC	Tetrahydrocannabinol
VTA	Ventral tegmental area

Introduction

What is the Nature of the Cognition of People with Low Levels of Striatal Dopamine?

The main abnormalities first associated with Parkinson's disease (PD) seem to be motor in nature: resting tremor, rigidity, slowed body movements, and shuffling gait. It is, therefore, not surprising that the discovery that PD patients present a massive loss of dopaminergic neurons of the

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substantia nigra pars compacta (SNc) (Bernheimer et al. 1973) was taken as strong evidence that dopamine is critical to movement. In PD, the loss of dopamine was most pronounced in the dorsolateral putamen, which apparently also implicates the dorsal striatum with movement control. This theory is also supported by the impairment in movement control observed in Huntington's disease (HD) patients, since a huge loss of medium spine neurons in the dorsal striatum is observed in this disease (Hooss and Margolis 2002).

However, more recently, a growing body of evidence also implicated PD and HD in cognitive functions (Montoya et al. 2006; Grahn et al. 2009). Subtle cognitive impairments can be observed even during the early phases of PD. They comprise a dysexecutive syndrome and working memory impairment accompanied by secondary deficits in the internal representation of visuospatial stimuli and in the use of declarative memory storage (Bradley et al. 1989; Owen et al. 1993; Dubois and Pillon 1997; Tamaru 1997; Goldman et al. 1998; Bosboom et al. 2004). Declarative memory per se is mostly preserved, but as discussed below it may be impaired in dementia, a condition that affects 20% or more PD patients (Brown and Marsden 1984; Aarsland et al. 1996; Zgaljardic et al. 2004).

Executive functions comprise a wide range of cognitive functions required for goal-directed, adaptive behavior in response to new, challenging environmental situations, including planning, task management, attention, behavioral monitoring, inhibition of improper behavior, and coding. All these functions are attributable to the prefrontal cortex, and therefore, PD cognitive disabilities resemble cognitive deficits found in patients with frontal cortex lesions (Tamaru 1997; Rogers 1998; Marie et al. 1999; Rowe et al. 2002; Burges and Alderman 2004; Owen 2004; Slabosz et al. 2006). Such impairments compromise the performance in initiating and planning daily tasks as well as in the execution of those that demand attention. They also show impairment in alternating two concomitant tasks according to the degree of importance and priority, in time estimation, and in solving tasks with chronological sequencing.

Working memory (Stebbins et al. 1999; Marie and Defer 2003), especially spatial working memory (Pillon et al. 1996, 1997; Owen et al. 1997), also fails in nondemented PD patients. The articulatory (verbal-phonological) component of the working memory is usually preserved, but when a verbal working memory task demands more attention, such a deficiency is also observed (Moreaud et al. 1997; Owen 2004). Such impairments are possibly the consequence of failure of the central executive component that manages the short-term memory. Thus, these impairments appear when the working memory tasks present a higher demand on executive functions such as planning and attention shifting (Grossman et al. 2003; Bosboom et al. 2004).

Procedural memory deficits are also reported to occur in PD, affecting more severely the initial learning phase

(Knowlton et al. 1996; Dujardin and Laurent 2003). Procedural memory is the knowledge of how to do something. Skills are an example of procedural memory affected by PD. Since many skills are closely related to motor functions, people used to see them as part of the motor disabilities of the disease. However, skills are cognitive abilities acquired by learning. Furthermore, the learning of more obviously cognitive skills is also affected in PD. These include reading mirrored image words, puzzle assembly, pressing specific keys on a keyboard in response to a stimulus presented on the computer screen, and drawing lines in hidden mazes (Bondi and Kaszniak 1991; Thomas et al. 1996; Moreaud et al. 1997; Koenig et al. 1999). In addition, other types of procedural memories are impaired even in nondemented PD patients. Habit is a clear example (Knowlton et al. 1996).

Habits are learned unconsciously by the repetitive association of a stimulus with a reinforced response. After extensive repetition, the response will be automatically triggered by the associated stimulus, independently of the outcome. Habit learning also contributes to drug addiction, as will be discussed later. Many of our daily jobs, like driving a car, turning right or left to go from home to work, using the grammatical rules of a language, or remembering that $2 \times 2 = 4$, are done automatically by habit. The main difficulty in modeling a habit task is to be sure that the subjects will not respond consciously to receive the reward, instead of responding in an automatic way. This question was addressed in a study by Knowlton and colleagues (1996) by testing whether nondemented PD patients fail to learn a probabilistic classification task. The probabilistic structure of the task permitted the subjects to learn the task unconsciously by trial-and-error. PD patients scored worse than Alzheimer's disease patients and healthy subjects, but when asked about it, they remembered having participated in the previous training sessions. Alzheimer's disease patients, on the other hand, learned this task like healthy subjects, but barely remembered the training episode.

It is not consensual that PD spares declarative memory (Bondi and Kaszniak 1991; Thomas et al. 1996). Declarative memory refers to the knowledge about facts, including the place they occurred, names of people involved, dates, etc. A general knowledge of the world, e.g., concepts, is also considered to be a kind of declarative memory. It is the knowledge of what was done, or what something is, while nondeclarative (procedural) memory is the knowledge of how to do something. PD patients are generally not impaired in encoding and storing declarative information, but they present difficulties in retrieving such information, particularly when they have to self-initiate remembering strategies (Dujardin and Laurent 2003). A failure in executive functions may explain this deficit. In addition, the nonintentional and automatic nature of a task, such as learning a list of words or matching pairs of words, may also determine

whether it can be learned normally by PD patients (Faglioni et al. 1995, 1997; Roncacci et al. 1996). Some authors explain that the declarative deficits reported in studies involving PD patients result from the fact that they require a larger number of repetitions of the task to translate procedural knowledge into declarative knowledge (Pascual-Leone et al. 1993).

The risk of developing dementia is up to six times higher in PD patients than in healthy subjects of the same age (Aarsland et al. 1996). The core of the impairments lies in executive functions (e.g. set-shifting) (Girotti et al. 1986). Mood disorder (e.g. depression), and psychotic (e.g. visual hallucinations) symptoms are also common in demented PD patients. Other common impairments include visuospatial and visuoconstructive skills. Speech and language difficulties, such as naming and sentence comprehension, are also common (Bosboom et al. 2004). Furthermore, poor verbal fluency would be predictive of dementia in PD. Declarative memory impairments are present, but are less severe, compared with Alzheimer's disease. There is a deficit in free recall, but it can be compensated for by semantic cueing. In addition, PD patients have more problems to recall than to encode declarative memories, i.e., their impairment relies on difficulties in activating processes involved in the functional use of memory storages, probably as a consequence of the dysexecutive syndrome (Dujardin and Laurent 2003; Bosboom et al. 2004). Recognition memory is relatively intact (Bosboom et al. 2004). Some of these cognitive impairments, especially attention impairment, are aggravated by a degeneration of cholinergic neurons in the nucleus basalis of Meynert and of noradrenaline neurons in the locus coeruleus that also occur in PD. On the other hand, impairments in declarative memory, aphasia and apraxia, when present, are usually related to cortical pathology indicative of Alzheimer's disease or Lewy body dementia (McKeith and Burn 2000; Tröster and Woods 2003). Regarding the last comorbidity, it is noteworthy that many characteristics of PD dementia resemble Lewy body dementia. Additionally, postmortem studies have revealed that many cases of Lewy body dementia had been wrongly diagnosed in life as PD and many PD patients develop Lewy body dementia later on (Zgaljardic et al. 2004).

From Movements to Actions

According to the evidence presented in the previous section, PD has been historically seen as a movement disease, but it also causes clear cognitive impairments that appear even before the motor disabilities can be noticed. Researches on HD have led to a similar picture (Montoya et al. 2006). When considering these findings, some influential behavior researchers (Mink 1996; Redgrave et al. 1999; Grillner et al. 2005; Hikosaka 2007; Redgrave et al. 2008) presented an

alternative hypothesis to explain the motor impairments of PD: patients are impaired in initiating actions rather than simple movements. Most actions depend on movements to be expressed, but an action is something more. It is a purposeful attitude directed toward a goal. Sitting down or standing up are actions. The coordinated sequence of muscle flexions and extensions are the movements needed to implement these actions. The same movements can be performed separately in a nonpurposeful manner. The same can be said in relation to crossing a road, picking an apple, eating a cake, and so on. All these examples of actions are part of the immense repertoire of the human behaviors that are carried out with an objective. All these actions depend on movements to be carried out, but other actions, like not crossing the road at a red sign or waiting for a bus, can be expressed as choosing not to move. They are expressed by a particular pattern of activity of postural muscles.

Therefore, if a critical level of striatal dopamine is needed to initiate the proper actions in particular instances, no movement will be seen in a PD patient, because the action that they put into operation is not being activated. This theory is consistent with the clinical reports of PD patients. They are usually impaired in starting an action, like walking, standing up, and so on. Once they start an action, they appear not to have the same difficulty in continuing. Sometimes they are impaired in finishing this action (Fahn and Przedborski 2000). In this sense, it can be said that a proper level of striatal dopamine is needed to choose and/or implement the choice of the proper action at the proper time.

The Basal Ganglia as an Action-Selection Device

One view accepted by many researches is that the basal ganglia form a system selecting appropriate actions under specific circumstances (Frank 2005; Balleine et al. 2007; Lau and Glimcher 2007). Appropriately choosing between distinct courses of actions requires the ability to integrate an estimate of the causal relationship between an action and its consequences, or outcome, with the value, or utility, of the outcome. The kind of learning that led to increasing the frequency of actions that resulted in a rewarding outcome and decreasing the frequency of those with aversive consequences was named instrumental, operant conditioning, or simply goal-direct learning. Sometimes the environmental cues (stimulus) that signal that an action will present rewarding or aversive consequences are also learned. After extensive training, the cue triggers the action in an automatic way, as if the subject were anticipating the outcome of his action before it can be evaluated. This kind of behavior is called stimulus-response habit.

Behavioral researchers have identified forms of learning in rodents homologous to goal-directed and habitual learning in humans. This suggestion is based on extensive evidence that choice between different actions is determined by the animal encoding the association between a specific action and outcome and the current value of the outcome (Balleine et al. 2007). Such research points to the striatum and the nigrostriatal pathway as critical to associative learning.

Studies in humans corroborate the research in animals suggesting that the dorsal striatum is an integral part of a circuit involved in learning and evaluating the reward consequences (O'Doherty et al. 2004; Tricomi et al. 2004). Accumulating evidence, primarily from neuroimaging but also from neuropsychological investigations, has implicated the dorsal striatum in different aspects of motivational and learning processes that support goal-directed actions. For instance, positron emission tomography (PET) studies report increases in dopamine release in the dorsal striatum when participants are presented with potential rewards, such as the opportunity to gain money (Koeppe et al. 1998; Zald et al. 2004) or when presented with food stimuli while in a state of hunger (Volkow et al. 2002).

Another kind of associative learning in which the nigrostriatal pathway takes part is the Pavlovian conditioning (Bayer and Glimcher 2005). In this kind of learning, the subject learns to anticipate a rewarding or aversive stimulus (unconditioned stimulus) by the presentation of a cue (conditioned stimulus).

Nowadays, many theories compete for the explanation of what the critical role of the basal ganglia is, specially the striatum and the midbrain dopaminergic neurons, in this kind of associative learning. One of these theories, called “the mosaic of broken mirrors model,” was proposed by us (Da Cunha et al. 2009). According according to this theory, the striatum receives information of parts of the surrounding environment (e.g. objects) with which parts of the subject's body (e.g. an arm, a finger, a foot) can interact. Each body part is encoded in the striatum in a functional manner – neurons representing a finger respond to the tactile perception of it as well as to the contractions of the muscles that move it. The representation of the body is not continuous as in the somatosensory and motor cortex, but fragmented. The surrounding environment is also represented in a polymodal way, fragmented in objects that a body part can interact with and locations the subject can move to. The units representing body parts, objects, and locations are redundant – these elements are represented repeatedly, like a mosaic made of broken mirrors. The repeated and widespread distribution of these units amplifies the combinatorial power of the associations among them. These associations depend on the phasic release of dopamine in the striatum triggered by salient stimuli and will be reinforced by the rewarding consequences of the actions related to them. Dopamine permits

synaptic plasticity in the corticostriatal synapses. The striatal units encoding the same stimulus/action send convergent projections to the internal segment of the globus pallidus (GPi) and to the substantia nigra pars reticulata (SNr) that stimulate or hold the action through a thalamus-frontal cortex pathway. According to this model, this is how the basal ganglia select actions based on environmental stimuli and store adaptive associations as procedural memories such as motor skills, habits, and memories formed by Pavlovian and instrumental conditioning.

There is compelling evidence in favor of this model. In the early 1990s, Graybiel and coworkers found evidence of redundant units representing body parts in the putamen of monkeys (Flaherty and Graybiel 1991). She called them *matrisomes*. In the following years, the view that the corticostriatal projections are parallel and segregated (Alexander et al. 1986) prevailed over that finding. However, more recently, the concept of *matrisomes* was resuscitated by reports of convergent and overlapping corticostriatal projections, including regions beyond the somatosensorimotor areas such as the prefrontal (Selemon and Goldman-Rakic 1985; Calzavara et al. 2007), posterior parietal (Cheatwood et al. 2005), secondary visual (Cheatwood et al. 2005), and cingulate cortex (Zheng and Wilson 2002), among others.

Another seminal study that influenced us to propose “the broken mirrors model” was also published in the 1990s by Graziano and Gross (1993). They reported that neurons of the putamen of monkeys presented tactile and visual receptive fields covering different body parts. Neurons responsive to the touch of a cotton swab in a monkey's face while its eyes were covered, increased their firing after the animal had its eyes uncovered, so that it could see this object approaching its face. The same neuron did not respond before the object was 10 cm or less from the animal's face. Coherent with our theory that these striatal neurons encode objects that can be manipulated by a body part, when the arm of the animal was moved out of its vision, a typical “arm + vision neuron” no longer responded to the presence of the object to its field of view. Based on these findings, they propose that the striatum encodes objects located in the visual space surrounding the subject in body-part, rather than in retinotopic coordinates. The proposal that the striatum also encodes locations is strongly supported by the electrophysiological studies of Mizumori and coworkers reporting place-related cells in the striatum similar to those previously found in the hippocampus (Mizumori et al. 2004, 2009).

The inputs to the striatum are not restricted to sensory, spatial, or motor areas of the cortex. Prefrontal and limbic areas of the cortex also project to the striatum in a convergent and widespread manner. Convergence refers to afferents departing from different regions of the cortex to overlap in restricted areas of the cortex forming “*matrisome-like*” functional units. These units are widely distributed in vast

regions of the striatum. Their function may be the association of abstract information such as symbols, words, digits, thoughts, and plans. The processing of these functional units by the basal ganglia would explain their involvement in working memory, executive, and emotional functions (Chang et al. 2007).

The association of the functional units of the striatum seems to be critical to associative learning. They form memories that are long-lasting due to synaptic plasticity processes, like long-term potentiation (LTP) and long-term depression (LTD), both reported to occur in the corticostriatal synapses (Calabresi et al. 2007). The release of dopamine in the striatum increases the probability of these processes (Di Filippo et al. 2008). This explains the many reports that dopamine is critical to associative learning (Da Cunha et al. 2003). The tonic release of dopamine in the presence of salient stimulus may be the learning signal that allows the occurrence of the synaptic plasticity processes necessary to change the connectivity among the functional units of the striatum.

Looking for the Specific Role of Dopamine in Reinforced Associative Learning

Midbrain neurons release dopamine in the striatum in tonic or phasic patterns (Goto et al. 2007). A small amount of dopamine is spontaneously and continuously released by these neurons in a tonic pattern, providing the baseline level of extrasynaptic dopamine required to run the motor programs already set up. Without this basal level of dopamine, even the choice of actions previously associated to predictive stimuli fails to be triggered as it occurs in PD (see above). It is the phasic firing of dopaminergic neurons that causes a transient and robust release of dopamine that serves as a learning signal, inducing neural plasticity in the striatum.

However, there is no consensus as to the nature of the critical stimuli that trigger the phasic release of dopamine or as to what the precise role played by the dopamine in reinforced learning is. There are three competing hypotheses: (a) the hedonia hypothesis of reward pleasure; (b) the reward learning hypothesis of associative stamping-in, teaching and predicting error; and (c) the incentive salience hypothesis of reward “wanting” (Berridge 2007). All the three suggestions form basic the components of reinforced learning: liking, learning, and wanting.

The hedonia hypothesis was proposed in the late 1970s by Wise (1980) and became the most influential view. According to this, the dopamine release, particularly into the nucleus accumbens (NAc), a ventral part of the striatum, signals the reward (liking). This view was modified in the reward prediction error theory proposed by Schultz (1998). According to this, the phasic release of dopamine occurs in response

to unpredicted rewarding stimuli, the amount of dopamine released being proportional to the difference between expected and obtained reward.

More recently, this theory has been contested by the argument that the latency for a stimulus to induce the phasic release of dopamine is too short to permit the sensory processing necessary to evaluate the hedonic value of the stimulus (Redgrave et al. 2008). The fact that the unpredicted presentation of nonrewarding salient stimuli such as light flashes or tones also elicits a phasic dopamine response also disagrees with the reward prediction error theory. New evidence suggests that the phasic dopamine response signals the presence of new biologically significant stimuli, with a positive response (increased release of dopamine) to nonharmful stimuli (neutral or rewarding) and a negative response to harmful stimuli.

The hypothesis that the phasic response of dopamine can trigger an action previously reinforced has also been contested by the finding that many phasically active striatal neurons respond at too late a time to initiate or select movements. Lau and Glimcher (2007) found that the encoding of action and outcome is performed by largely separate populations of caudate neurons that are active after movement execution. Thus, striatal neurons, active primarily after a movement, appear to be segregated into two distinct groups that provide complementary information about the outcome of actions (one population encodes information about the movement just executed, whereas the other encodes the received outcome).

Like dorsal striatal neurons, NAc neurons also exhibit premovement firing after the instruction in goal-directed action tasks (Schultz et al. 1992; Bowman et al. 1996; Nicola 2007). However, it seems that the dopamine requested by NAc during the action selection depended whether the previous event and the cue presentation were short (<10 s and usually ≤ 5 s) or whether the interval was longer (often >15 s). It appears, then, that NAc dopamine is required for responding to stimuli presented after a long interval, since the animal had to perform an action (“long-interval stimuli”), but not for cues presented a short time before the previous action (“short-interval stimuli”) (for more detail see Nicola 2007). In the Nicola’s model (2007), it was proposed that different NAc neurons represent different stimulus–action associations and that when the stimuli activating these representations are perceived simultaneously, the neurons compete with each other for control over behavior. Dorsal striatal neurons clearly represent stimulus–action associations, and several lines of evidence support the idea that associations between temporally unpredictable stimuli and actions are represented by NAc neurons and control behavior in response to such stimuli (“NAc and action selection”). However, a critical piece of evidence in favor of this hypothesis is still lacking: a demonstration that the stimulus-evoked firing of NAc neurons encodes specific

movements. Nevertheless, the wealth of evidence indicating that this is the case for dorsal striatal neurons suggests a similar role for NAc neurons. In this case, the dopamine would facilitate this process by increasing the firing of those NAc neurons representing the most beneficial stimulus–action association and increasing the probability that the represented action will occur. The circuit underlying this competition is supposed to utilize direct and indirect output paths from the NAc, similar to those used by dorsal striatal neurons. This circuit provides a specialized mechanism for controlling action in response to unpredictable stimuli, which may serve to set the animal’s overall course of action (see [Da Cunha et al. 2009](#) for more details on the role of the direct and indirect corticobasal pathways on learning and action selection).

The incentive salience hypothesis is built on earlier incentive theory formulations on motivation and on the role of dopamine. It suggests that dopamine mediates the “wanting” but not the “liking” or “learning” components of rewards. According to this hypothesis, it transforms the brain’s neural representations of conditioned stimuli, converting an event or stimulus from a neutral “cold” representation (mere information) into an attractive and “wanted” incentive that can grab attention.

Incentive salience is also motivational because its attribution transforms the neutral representation of a stimulus into an object of attraction that the animal will work to acquire ([Berridge and Robinson 1998](#)). When incentive salience is attributed to the reward-related stimulus, it transforms the brain’s representation from a mere perception or memory into a motivationally potent incentive. Whether attributed to an unconditioned reward or to a conditioned stimulus that predicts reward, incentive salience makes those stimuli more attractive and “wanted.” The neural machinery responsible for this attribution involves dopamine neurotransmission as one link in a larger chain of mesocorticolimbic circuits and signals ([Berridge 2007](#)).

One consequence of incentive salience attribution is that a conditioned stimulus or a cue for rewards themselves can become a motivational magnet, capable of triggering further “wanting” for their reward. This happens because the conditioned stimulus can take on certain incentive motivational properties of its reward via associatively guided attributions, thus becoming “wanted” and “liked” as much as the reward itself (though “liking” and “wanting” features have separable neural substrates both for conditioned stimulus and reward). Dopamine activation or suppression specifically modulates the strength of this cue-triggered burst of “wanting” motivation, which decays away soon after the reward cue is removed, only to reappear again when the cue is encountered later ([Wyvell and Berridge 2001](#); [Berridge 2007](#)).

There are three stages involved in incentive salience. The first time the unconditioned hedonic pleasure (“liking”) is encountered, it acts as the normal trigger for the reward-building process, and activates the associative and incentive salience steps. But “liking” by itself is not sufficient to motivate behavior. The second time happens when associative learning systems target incentive salience attributions to conditioned stimuli associated with the “liked” reward. The last stage happens on subsequent occasions when incentive salience is attributed to conditioned stimuli by the activation of dopamine-related systems, guided by associative learning, making the conditioned stimulus a target of “wanting” and a trigger of increased “wanting” for its reward. The conditioned stimuli can also activate conditioned “liking” via separate hedonic brain systems other than dopamine ([Berridge and Robinson 1998](#); [Berridge 2007](#)).

Incentive salience attribution is strongly modulated by reward-relevant physiological states of an individual at the moment a stimulus is encountered ([Berridge and Robinson 1998](#); [Berridge 2007](#)). In other words, the incentive salience of a cue for a food reward is extremely amplified by physiological hunger states. Animal experiments made by Petrovich, in 2005, have shown this as conditioned appetite and may involve a linked activation of limbic and hypothalamic systems. This phenomenon is highly specific: relevant reward stimuli become even more “wanted,” but other irrelevant reward stimuli are relatively unaffected. ([Berridge 2007](#)).

To discriminate the cue incentive salience (“wanting”) from the cue learned predictions (and reward learning prediction errors) or from hedonic impact of reward (“liking”) [Tindell and colleagues](#), in 2005, used two serial conditioned stimuli that predicted a sucrose reward. The first was a ten-second auditory tone that carried the highest learned reward prediction value, because it invariably signaled the rest of the series (conditioned stimulus 2 and then the reward – prediction was always 100% in this study). The second conditioned stimulus was a one-second auditory click and carried the highest incentive salience, since it was a marker of immediate reward. Finally, the reward, which consisted of a sucrose pellet, arrived within one second after the second stimulus, and hence, it probably carried the highest hedonic impact (“liking”) or prediction error. Electrophysiological results showed that both amphetamine treatment and neural sensitization specifically amplified incentive salience as verified in elevated peaks of neuronal firing triggered by the conditioned stimulus 2, which immediately preceded the reward.

Complementarily, [Wyvell and Berridge \(2001\)](#) found out that the power of conditioned stimulus presence is determinant to the dopamine activation, because there is a synergy of enhancements of incentive salience, in that both dopamine activation and conditioned stimulus presence seem to be required simultaneously.

It is still too early and the evidences found until now are too little to draw final conclusions, but so far, these and other data suggest that manipulations that enhance dopamine neurotransmission can amplify the mesolimbic transformation of learned signal into incentive salience that gives them motivational values, without amplifying either “learning” computational parameters or hedonic “liking” (Berridge 2007).

The Role of Dopamine in Dependence and Addiction

The involvement of dopamine in reinforced learning and rewarding processes is implicated in drug addiction. Many groups working in that field consider the mesolimbic dopamine system and its forebrain targets as part of the motivational system that regulates responses to natural rewards, such as food, drink, sex, and social interaction. Drugs of abuse affect this pathway with a strength and persistence probably not seen in response to natural rewards (Nestler 2001). It has been hypothesized that addictive drugs have a competitive advantage over most natural stimuli in that they can produce far greater levels of dopamine release and more prolonged stimulation. Thus, dopamine is thought to have an important role in the habit-forming actions of several addictive drugs (Wise 1978; Wise and Bozarth 1987; Di Chiara and Imperato 1988).

Drug Dependence refers to a state resulting from habitual drug use and the word dependence has multiple meanings. Any drug can produce dependence if dependence is defined only taking in consideration the manifestation of a withdrawal syndrome upon cessation of drug use. Using the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM IV) (American Psychiatric Association 1994) criteria, substance dependence is beyond the manifestation of a withdrawal syndrome, but rather equivalent to addiction. Dependence is generally used to refer as chronic, compulsive, and uncontrollable drug use, to the extent to which a person (an “addict”) cannot or will not stop the use of some drugs, known as “loss of control.” It usually implies psychological and physiological components, the last resulting in a withdrawal syndrome when drug use is stopped abruptly (Le Moal and Koob 2007). Among DSM-IV criteria, we can define drug addiction as a chronically relapsing disorder characterized by compulsion to use one or more drugs of abuse, the inability to control drug intake, and continued drug use despite negative consequences (Leshner 1997; Deroche-Gamonet et al. 2004).

Addiction is a complex disorder, affecting not only the individual but also their family and the community at large (Lawrence et al. 2008). Thus, addiction continues to exact enormous human and financial cost on society, but available

treatments remain inadequate and inefficient for most people (Nestler 2001). Addiction has been conceptualized as a progression from impulsive to compulsive behavior, ending in chronic, relapsing drug taking. Addiction is caused by the actions of a drug of abuse on a vulnerable brain and generally requires repeated drug exposure. This process is strongly influenced both by the genetic makeup of the person and by the psychological and social context in which drug use occurs. Once formed, addiction can be a life-long condition in which individuals show intense drug craving and increased risk for relapse after years or even decades of abstinence. This means that addiction involves extremely stable changes in the brain that are responsible for these long-lived behavioral abnormalities (Nestler 2001).

Addiction is proposed to involve three stages: binge/intoxication, withdrawal/negative effect, and preoccupation/anticipation. In the first stage, the drive for the drug-taking behavior is the positive reinforcement, in which stimuli increase the probability of the response and the impulsive behavior predominates. In the second stage, the drive transition to negative reinforcement occurs and the individual returns to the use of the drug to remove the withdrawal-induced aversive state. In the last stage, the compulsion is installed, and the loss of control is observed. Different theoretical perspectives from experimental psychology (positive and negative reinforcement framework), social psychology (self-regulation failure framework), and neurobiology (counteradaptive and sensitization framework) can be superimposed on the stages of the addiction cycle. These stages interact with each other leading to the pathological state known as addiction (Koob and Le Moal 2005).

For the binge/intoxication stage, studies on the acute reinforcing effects of drugs of abuse “per se” have identified key neurobiological substrates. Important anatomical circuits include the mesocorticolimbic dopamine system originating in the ventral tegmental area (VTA) and projecting to the NAC and the extended amygdala. One likely mechanism of addiction, then, is that repeated, strong stimulation of the mesolimbic dopaminergic neurons changes them in a way that leads to marked alterations in reinforcement mechanisms and motivational state. Several types of functional alterations have been described (Koob and Nestler 1997; Nestler 2001).

There is strong evidence that drugs of abuse that produce reward, cause reinforcement and may cause dependence. They also increase dopamine release in mesolimbic regions. These drugs include opiates, cannabis, and nicotine, as well as drugs that act directly on presynaptic dopamine terminals (amphetamine, methamphetamine, and cocaine) (Marsden 2006). However, phencyclidine, morphine, and nicotine, at least, seem to have dopamine-independent as well as dopamine-dependent rewarding effects (Wise 2004).

Although drugs of abuse are chemically divergent molecules with very different initial actions, the resultant

addictions share many important features. This can be explained by the fact that each drug, despite its many distinct mechanisms of action in the brain, converges into producing some common actions. Prominent among these actions is the activation of the mesolimbic dopamine system. This activation involves an increased firing of dopamine neurons in the VTA of the midbrain and a subsequent increase in dopamine release into the nucleus accumbens (NAc) and other regions of the mesolimbic forebrain (for example, the prefrontal cortex). Several drugs of abuse also activate the mesolimbic dopamine system indirectly by mimicking (opiates) or activating (alcohol, nicotine) endogenous opioid pathways that innervate VTA and NAc (Nestler 2001).

Psychostimulants such as cocaine and d-amphetamine elevate extracellular dopamine by inhibiting the reuptake of dopamine by the dopamine transporter and in the case of d-amphetamine, also by promoting reverse transport of dopamine (Koob et al. 1998). Besides inhibiting the dopamine transporter, psychostimulants also inhibit the reuptake of serotonin and noradrenaline, which may contribute to their reinforcing actions; possibly, in part, by modulating dopamine neurotransmission (Parsons et al. 1995).

The rewarding properties of the opiates appear to be largely mediated by μ opioid receptors, since selective μ antagonists decrease opioid reinforcement in a dose-dependent manner (Negus et al. 1993). Thus, opiate-induced reinforcement utilizes the same circuitry implicated in the actions of stimulants like cocaine and amphetamine, but may involve additional sites of interaction (Koob and Bloom 1988). The blockade of opioid receptors, either in the VTA or in the nucleus accumbens, will decrease heroin self-administration (Koob et al. 1998). Opiates, like other drugs of abuse, increase dopamine release in the nucleus accumbens (Di Chiara and Imperato 1988). However, the reinforcing effect of opiates in the nucleus accumbens persists even when all the dopamine projections are destroyed, suggesting that their reinforcing actions may involve both dopamine-dependent (VTA) and dopamine-independent (nucleus accumbens) mechanisms (Koob and Bloom 1988).

Ethanol reinforcement also appears to involve the activation of brain dopamine systems. Acute ethanol consumption or systemic injection reduces the firing rate of pars reticulata gamma amino butyric acid (GABA) neurons, which are thought to exert an inhibitory control over VTA dopaminergic neurons (Diana et al. 1993). A growing body of evidence indicates that ethanol enhances dopamine release in the nucleus accumbens (Di Chiara and Imperato 1988; Yim and Gonzales 2000). In addition, ethanol, by promoting GABA-A receptor hyperfunction, may inhibit GABAergic terminals in VTA and hence may disinhibit VTA dopamine neurons (Nestler 2005).

Intravenous self-administration of nicotine is also blocked by dopamine antagonists and dopamine-selective

lesions of the nucleus accumbens (Corrigal et al. 1992). Besides, nicotine seems to activate VTA dopamine neurons directly via stimulation of nicotinic cholinergic receptors localized on those neurons and indirectly via stimulation of its receptors on glutamatergic nerve terminals that innervate dopamine cells (Nestler 2005).

Tetrahydrocannabinol (THC) shares effects in animal models of drug reinforcement similar to those of other drugs of abuse (Anthony et al. 1994). Moreover, THC activates the mesocorticolimbic dopamine system (Chen et al. 1991) and increases the release of dopamine in the shell of the nucleus accumbens (Tanda et al. 1997).

As already mentioned, the VTA-NAc pathway is one of the most important substrates for the acute rewarding effects of all drugs of abuse, and research over the past several decades has delineated how each drug, regardless of its distinct mechanism of action, converges on the VTA and NAc with common acute functional effects. Each drug activates dopaminergic transmission in the NAc and many produce dopamine-like, yet dopamine-independent, effect on the same NAc neurons, in many cases via indirect, circuit-level actions (Koob and Le Moal 2001; Nestler 2001, 2005; Wise 2004).

More recent work has established that several additional brain areas that interact with the VTA and NAc are also essential for acute drug reward and chronic-induced changes in reward associated with addiction. These regions include the amygdala (a related structure of the so-called "extended amygdala"), hippocampus, hypothalamus, and several regions of the frontal cortex, among others (Hyman and Malenka 2001; Koob and le Moal 2001; Nestler 2001). Interestingly, some of these areas are part of the brain's traditional memory system; which has led to the notion, now supported by increasing evidence, that important aspects of addiction involve powerful emotional memories (Nestler 2001).

Over the past few years, several groups have documented that repeated exposure to a drug of abuse causes structural changes in specific neuronal cell types that are implicated in the second stage of addiction cycle and induce plasticity in neural circuitry that drives compulsive drug taking (Nestler 2001). Just as all drugs of abuse increase dopaminergic transmission to the NAc after acute injection, they also produce common adaptations in dopamine function after chronic exposure (Nestler 2005). Chronic exposure to any of several drugs of abuse causes an impaired dopamine system, which can be viewed as a homeostatic response to the repeated drug activation of the system (in other words, tolerance). After chronic drug abuse, baseline levels of dopamine function are reduced, and normal rewarding stimuli may be less effective at eliciting typical increases in dopaminergic transmission (Nestler 2005). A change in the pattern of dopamine neurons firing is also observed. Under

basal circumstances, dopamine neurons exhibit a relatively consistent tonic pattern of firing, superimposed by brief phasic bursts of spike activity when an unexpected reward is presented. As the individual learns that certain signals (like a tone or light) predict this reward, the timing of this phasic activity changes and then the predictive stimulus causes phasic bursts. If a stimulus normally associated with a reward is presented but the reward is withheld, there is a pause in the tonic firing of dopamine neurons at the time that the reward would have been expected. In contrast, if a reward comes at an unexpected time or exceeds expectation, a phasic burst in firing is observed. It has been hypothesized that these phasic bursts and pauses encode a prediction error signal. Tonic activity signals no deviation from expectation, but phasic bursts signal a positive reward prediction error (better than expected), and pauses signal a negative prediction error (worse than expected). The direct and indirect pharmacological actions of addictive drugs increasing dopamine levels that would not decay over time would predict an additional advantage for drugs over natural rewards. Thus, the brain would repeatedly get the signal that drugs are “better than expected” (Hyman 2005).

Moreover, significant plasticity occurs in the neurotransmitter circuits identified earlier as critical to the acute reinforcing effects of drugs of abuse. In animal models of transition to addiction, there occur changes in brain reward threshold that temporally precede and highly correlate with escalation in drug intake (Ahmed et al. 2002). There is a decreased activity of the mesocorticolimbic dopamine system as measured by electrophysiological recordings and in vivo microdialysis and also decreased activity in opioid peptide, GABA, glutamate, and neuropeptide Y (Koob and Le Moal 2005). While the rewarding effects accompanying the acute administration of most drugs of abuse depend on increased dopamine release in the accumbens (Wise and Rompre 1989; Koob and Le Moal 2001), the reinstatement of drug seeking requires dopamine release in the prefrontal cortex and amygdala (McFarland and Kalivas 2001; See et al. 2001; Capriles et al. 2003; McFarland et al. 2004), not in the nucleus accumbens core (Cornish and Kalivas, 2000; Di Ciano et al. 2001; McFarland and Kalivas 2001). Dopamine release in the prefrontal cortex is antecedent to the activation of the projection from the prefrontal cortex to the accumbens core, since preventing cortical dopamine release prevents glutamate release in the nucleus accumbens by a stress or drug prime (McFarland et al. 2003, 2004).

Numerous types of drugs of abuse, including cocaine, amphetamine, opiates, ethanol, or nicotine, induce a long-term potentiation (LTP)-like state in VTA dopamine neurons (Kauer 2004). In addition, chronic administration of any of several drugs of abuse, including cocaine, amphetamine, opiates, alcohol, and nicotine, also increases levels of

tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, in the VTA (Nestler 1992; Lu et al. 2003).

From another point of view, at molecular levels, drugs of abuse promote many different effects in the basal ganglia. Although neural genes are probably regulated by hundreds of distinct types of transcription factors, two transcription factors in particular have so far been implicated in addiction: the cyclic-AMP response-element-binding protein (CREB) and FosB (Nestler 2001).

Moreover, evidence implicates the transcription factor CREB (cAMP response element binding protein) (Nestler 2005), which is also activated in VTA by several drugs of abuse after chronic administration, in mediating the drug induction of GluR1 (Olson et al. 2005) and TH in this region as well as in some of the behavioral plasticity associated with addiction (Walters et al. 2005).

CREB was first implicated in drug addiction, because its activation was a predictable consequence of the upregulation of the cAMP pathway, one of the best established adaptations to drugs of abuse (Guitart et al. 1992). Similarly, chronic morphine and cocaine treatments have been shown to enhance CREB function in NAc and striatal regions (Turgeon et al. 1997; Shaw-Lutchman et al. 2002).

Dynorphin causes dysphoria by decreasing dopamine release within the NAc through an action on Kappa-opioid receptors that are located on presynaptic dopamine-containing nerve terminals in this region (Hyman 1996; Kreek 1997; Shippenberg and Rea 1997). The expression of dynorphin is induced in the NAc and in related striatal regions after drug exposure. Moreover, CREB seems therefore to increase the gain on this dynorphin-mediated negative feedback circuit and thereby contributes to the generation of aversive states during withdrawal. An important goal of current research is to identify other CREB-regulated genes in the NAc and to relate them to specific features of drug dependence (Nestler 2001).

In the third stage of the addiction cycle (preoccupation/anticipation stage), a critical problem is relapse, in which addicts return to compulsive drug taking long after acute withdrawal. Several pieces of evidence show that the prefrontal cortex system (orbitofrontal, medial prefrontal, prelimbic/cingulate) and the basolateral amygdala are key mediators of drug- and cue-induced reinstatement in animal models and craving and relapse in humans. Neurotransmitter systems implicated in this stage include dopamine, opioid peptides, glutamate and GABA. Neuroplasticity in the natural reward system is highlighted by a decreased dopaminergic activity and hypofrontality (homeostasis). Neuroplasticity in the anti-reward system (neuroadaptations which produce aversive or stress-like states) is highlighted by an increased corticotropin-releasing factor (CRF) function (together with norepinephrine and dynorphin recruitment)

and is hypothesized to be particularly slow to return to homeostasis (Valdez et al. 2002; Koob 2003; Koob and Le Moal 2005).

In summary, dopamine plays an important role in dependence and in addiction, and seems to act as a maestro of this neurotransmitters orchestra involved in both phenomena. However, efforts must be made to understand the participation of each member cited earlier.

The Role of Dopamine in Depression

The dopaminergic system seems to be involved not only in cognition, reward, or drug addiction as discussed in the previous sections. Although depression has been generally associated with noradrenergic and/or serotonergic neurotransmission alterations, there are some data that also suggest a role for the dopaminergic system (Willner 1983; Dunlop and Nemeroff 2007). Depression associated with PD is frequently cited as an evidence for dopaminergic alterations contributing to depression (Willner 1983; Dunlop and Nemeroff 2007). However, although the main neuropathological finding in PD is the degeneration of nigrostriatal dopaminergic neurons, pathological changes in the locus coeruleus and raphe nuclei have also been found. These findings, associated with the clinical antidepressant efficacy of selective serotonin reuptake inhibitors and low level of 5-hydroxyindoleacetic acid in cerebrospinal fluid of these patients, have led to the hypothesis of serotonin dysfunction as the neurological basis of depression in PD patients (Mayeux 1990). On the other hand, recent studies have postulated a major role for dopaminergic alterations in depression associated with PD. For example, in a neuropathological study, Frisina et al. (2009) suggested a more catecholaminergic contribution than serotonergic to depression in PD patients. Unfortunately, the SN degeneration was almost 100% both in depressed and nondepressed PD patients, which makes it difficult to compare. However, the prevalence of gliosis in SNc was greater in depressed patients than in nondepressed patients (Odds Ratio=2.85). Another study observed that depressed PD patients have a blunted euphoric response to methylphenidate when compared with nondepressed PD patients, patients with major depression without PD, and controls (Cantello et al. 1989). Moreover, although bupropion, an antidepressant that inhibits noradrenaline and dopamine reuptake, exerts only a minor antidepressant effect in PD patients (Goetz et al. 1984), pramipexole, a direct DA receptor agonist, appears to have a significant antidepressant effect both in depressed PD patients (Barone et al. 2006) and depressed patients without PD (Zarate et al. 2004). Similarly, L-DOPA infusion improves mood in early PD patients (Maricle et al. 1998).

Although the main focus of DA role in depression is the VTA (e.g. Winter et al. 2007), a region associated to reward and pleasure, there are some data that also suggest the involvement of SNc. One line of evidence comes from case reports that found that unilateral (left or right) electrical stimulation of SN (or surrounding areas) induced acute depressive symptoms (Bejjani et al. 1999; Blomstedt et al. 2008). Depression (without PD) is also associated with hyperechogenicity in SN in transcranial sonography, a finding characteristic of idiopathic PD and regarded as a trait marker for SN vulnerability; depressed PD patients showed a greater frequency of this alteration than depression or PD (Walter et al. 2007).

Preclinical experiments also link depression and SNc. Winter et al. (2007) showed that concurrent chemical lesions of SNc and VTA lead to an increased depressive behavior in learned helplessness (indicated by increased latency and percentage of failure). Moreover, the increase in SNc lesion increases the depressive behavior, even when the length of VTA lesion is constant. It is interesting to note that these findings with unilateral lesion are in parallel with the deep stimulation case reports cited earlier. Pioli et al. (2008) observed that bilateral lesion of SNc impairs motivation (increases time to consume sweet pellets), although it also damages fine motor functions. On the other hand, VTA lesion did not change pellets consumption (Pioli et al. 2008). Also, Tadaiesky et al. (2008) observed that bilateral lesion of the striatum (which also causes dopaminergic cell loss of SNc) induces depressive behaviors as well as anhedonia (decrease in sucrose consumption) and increases behavioral despair.

Although some of the findings mentioned could also be associated with noradrenergic and/or serotonergic changes (e.g. Bejjani et al. 1999), taken together they indicate a putative role for SNc dopaminergic neurotransmission in depression.

Concluding Remarks

Dopamine, initially seen as part of a system involved in motor control, is nowadays recognized as critical to cognitive and emotional functions. Midbrain neurons, including those of the SNc, are part of a system that selects adaptive behaviors suitable for specific demands of the environment. A basal level of striatal dopamine is required for the selection of actions according to the outcomes signaled by environmental cues. The learning of new stimulus-response-outcome associations demands a phasic response of the midbrain neurons that results in a high increase of the dopamine level in the striatum. Such dopamine response seems to occur in response to new biologically salient stimuli. It is a kind of learning

signal, a signal that there is something new and relevant to be learned. At these situations, the synapses between the functional units of the striatum encoding the salient cues, the selected action, and the rewarding or aversive outcome will be strengthened or weakened, allowing the learning of these relations and thus affecting the future choice of actions. These functional units are distributed in the striatum as a mosaic of broken mirrors where the pieces represent body parts and objects or locations with which they can interact. The link of these pieces encodes skills, goal-directed actions, and stimulus–response habits. The link of other striatal units representing concepts, ideas, and other abstractions enable mental processes known as executive functions.

Due to the importance of these processes in which the dopamine plays a role, PD patients are impaired not only in the selection and initiation of motor actions, but also in cognitive and emotional faculties. Up to one third of them progress to dementia, and up to half of them develop depression. In addition, other cognitive and emotional impairments observed in other pathologies, like drug addiction, schizophrenia, and other mental diseases, are related to the malfunctioning of the midbrain dopaminergic system.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 13

The Substantia Nigra, the Basal Ganglia, Dopamine and Temporal Processing

Catherine R.G. Jones and Marjan Jahanshahi

Abstract It has been proposed that the basal ganglia are important to the temporal processing of milliseconds- and seconds-range intervals, both within the motor and perceptual domains. This review summarizes and discusses evidence from animal, pharmacological, clinical, and imaging research that supports this proposal, with particular reference to the role of the substantia nigra (SN).

Keywords Temporal processing • Basal ganglia • Substantia nigra • Parkinson's disease • Timing • Time estimation • Time reproduction • Dopamine • Frontostriatal circuits

Abbreviations

ADHD	Attention-deficit hyperactivity disorder
DBS	Deep brain stimulation
DA	Dopamine
PD	Parkinson's disease
SET	Scalar expectancy theory
SBF	Striatal beat frequency model
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SMA	Supplementary motor area

Introduction

Timing is an extremely broad-ranging topic. It encompasses circadian rhythms, the hard-wired 24 h cycles that govern sleep and feeding patterns and which are an essential

component of survival, through to the psychological conception of the passage of time in the order of weeks, months, and years, which relates to memory and a sense of self in humans. This broad field has garnered interest from a wide range of disciplines, including philosophy, biology, physiology, and psychology. A considerable body of research suggests that the basal ganglia and the neurotransmitter dopamine (DA) play a key role in temporal processing. Specifically, the basal ganglia are proposed to be important for the processing of time within the milliseconds-to-seconds range, both in the presence or absence of a motor component, respectively exemplified by clapping in time with music or perceptually detecting a rhythm change when listening to music. This type of timing is sometimes referred to as "interval timing" and is considered an essential component of human behavior. Temporal processing of this nature enables an individual to negotiate his/her environment accurately, from deciding when a kettle may have boiled through to judging when to reach to catch something. It is also critical in enabling individuals to share and participate in the same perceptual experience, for example, imagine an orchestra or a dance performance without a shared rhythm between participants. Research in this area often subscribes to the concept of a hypothetical "internal clock", posited to be located within the basal ganglia and the nigro-striatal dopaminergic system. In the following sections we review evidence from animal, pharmacological, clinical, and imaging research that suggests that the basal ganglia play a major role in this specific type of temporal processing.

For simplicity, hereafter, the term "temporal processing" refers specifically to motor and perceptual timing in the milliseconds and seconds range. It is also important to establish a theoretical framework for conceptualizing this type of temporal processing as a unitary construct. There is evidence from healthy participants that motor and perceptual timing abilities correlate (Keele et al. 1985; Merchant et al. 2008). Further, as will be discussed, difficulties with both types of timing are observed in patients with Parkinson's

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disease and functional imaging research has found common neural correlates. The range of durations covered by “interval timing” is not clearly defined and historically (and in part, by definition) motor tasks tended to use very short milliseconds-range intervals, whereas perceptual tasks tended to use longer intervals. Most experimental research into the psychophysiological and neurological components of the “internal clock” interpret data in terms of a three-process model of timing: a clock component that directly represents the passing of time (i.e. a pacemaker), a memory component that stores important clock values and a decision component that compares the current clock value with values stored in memory, to enable a temporal decision to be made. Models of temporal processing relevant to the basal ganglia will be discussed in more detail at the end of this review.

Experimental Studies of the Role of the Basal Ganglia and Dopamine in Temporal Processing

Animal Research

The most popular method for studying temporal processing in animals is the peak interval procedure. During fixed interval trials, a sound or light signal is introduced and the animal is rewarded with a food pellet when it presses the response lever after a fixed interval (e.g. 20 s) from the onset of the signal, with the lever press also terminating the signal. During probe or peak interval trials, the food reward is not made available after the fixed interval elapses and the signal will typically last at least three-to-four times the duration of the fixed interval. For these trials, a response-rate function is generated that plots the number of responses as a function of time from stimulus onset. The time at which maximum responding occurs is the peak time and reflects the animal’s judgement of the fixed interval. Another popular method, the temporal bisection task, trains the animal to discriminate between two durations (e.g. specified by a light or sound) of different lengths (e.g. 2 s and 8 s). The animal learns to pair the two intervals with different lever responses (e.g. right lever for a long interval; left lever for a short interval) in order to receive a food reward. During the testing phase the short and long intervals are presented along with unrewarded intervals of intermediate durations. The production of “long” responses are plotted to produce a (typically) sigmoidal-shaped curve, reflecting that the proportion of “long” responses increases as a function of stimulus duration. The bisection point (or point of subjective equality) is that duration at which long and short responses occur with equal probability. The temporal generalization task is

very similar to the temporal bisection task, with only the decision process differing. Through food reward, the animal is trained to press a lever after the presentation of an interval (e.g. light or sound) of a specific length (the criterion duration). Following this, the animal is presented with a variety of durations, including the criterion duration and both shorter and longer intervals. If the animal presses a lever after presentation of the criterion duration (typically occurring on 50% of trials) a food reward is delivered. No reward is delivered following a lever press in response to any of the other durations. The data can be plotted as a temporal generalization gradient, which illustrates the probability of a response as a function of signal duration, i.e., with responses peaking at the reinforced duration.

Investigations using pharmacological manipulation have shown that drugs that increase DA (e.g. methamphetamine, cocaine) lead to a horizontal leftward shift in the response curve, this suggests an underestimation of the interval being produced or judged (e.g. Cheng et al. 2007a, b; Maricq and Church 1983; Matell et al., 2004, 2006). Similarly, drugs that decrease DA transmission (e.g. haloperidol) lead to a horizontal rightward shift in the response curve, suggesting an overestimation of the temporal interval (e.g. Drew et al. 2003; MacDonald and Meck 2005; Maricq and Church 1983). Crucially, these temporal distortion effects are proportional to the duration being timed. The results are interpreted in terms of a respective speeding up (DA agonist effect) or slowing down (DA antagonist effect) of the “internal clock” and are linked to nigrostriatal-dopaminergic projections. However, it is also important to note that cholinergic drugs affect memory storage of intervals (e.g. Meck and Church 1987), such that the temporal processing network is unlikely to be purely carried in the domain of dopamine.

Lesion work has shown that ablations of the rat substantia nigra (SN) cause timing impairment, which is restored with administration of levodopa (Meck 2006). Damage to the caudate-putamen also affects discrimination, although this damage is impervious to levodopa treatment (Meck 2006a,b). Matell et al. (2003) recorded neuronal activity from the dorsolateral anterior striatum in rats during a temporal generalization task in which they learnt that a lever press response to two different durations (10 s and 40 s) could provide a food reward. They observed neurons that showed an increase in firing rate up to the time of the expected reward and then showed a gradual decrease in the firing rate thereafter. This is consistent with Matell and colleagues’ assertion that neurons in the striatum encode temporal durations as a function of their firing rate. Further, they reported neurons in the striatum and anterior cingulate that showed a difference in their firing rates during the two temporally defined periods of reward expectation. In primate research, increased striatal activity is observed during the delay preceding an anticipated stimulus (Apicella et al. 1992; Schultz et al. 1992).

Kobayashi and Schultz (2008) reported an increased response of DA neurons in the SN to a predicted reward when the delay before the reward was long: the reward delay varied from 2 to 16 s. They suggested that this could be related to the greater temporal uncertainty and difficulty in predicting the exact timing of more delayed rewards (i.e. leading to a greater temporal prediction error), particularly as reduced temporal precision is an inherent feature of increasing interval length (e.g. Gibbon 1977). Further, it has been shown that the response of DA neurons in the substantia nigra pars compacta (SNc) is modulated by an expected, but undelivered, reward as well as when a reward is delivered at an unexpected time (Hollerman and Schultz 1998). However, the same neurons do not fire when a reward occurs at a predicted time (at 1,000 ms after their response), again suggesting a role for DA in the coding of temporal errors in reward prediction.

Pharmacological Research

Pharmacological work in humans has also demonstrated a significant effect of DA on temporal processing. DA antagonists such as haloperidol and remoxipride, adversely affect duration discrimination (Rammsayer 1993, 1997). However, haloperidol affected both milliseconds- (50 ms standard) and seconds- (1,000 ms standard) range discrimination, whereas remoxipride only affected seconds-range discrimination (Rammsayer 1993, 1997). Thus, it is suggested that temporal processing in the millisecond-range is dependent on D2 receptor activity in the nigrostriatal system, a circuit that is not affected by remoxipride. Furthermore, it is proposed that the processing of seconds-range durations is dependent on D2 receptor activity in the mesolimbocortical system, a target of both drugs, which mediates memory functions. Rammsayer proposes that very short durations (such as the 50 ms used in his studies) are below the threshold for cognitive control and are dependent on dopaminergic activity in the basal ganglia. In contrast, seconds-range timing depends on the efficacy of working memory processes, with directed attention also being influential (e.g. Rammsayer 1999; Rammsayer et al. 2001). Levodopa, the precursor of DA, is used in the treatment of Parkinson's disease (PD) to increase levels of DA in the brain. In healthy adults, DA administration during performance of the human analog of the peak interval procedure has the effect of lengthening the reproduction of previously learned seconds-range intervals (6 and 17 s) (Rakitin et al. 2006). This is perhaps surprising, since from a straightforward model of the effects of DA on timing one would predict that increasing effective levels of DA would "speed" up the internal clock and therefore produce shorter reproductions. However, as the size of the error was similar at

both durations (and not proportional), this does not suggest that "clock" speed has been affected. Rather, it is suggested that the process of switching attention to time has been slowed, i.e., the "switch" process within the internal clock has been affected. Similarly, DA agonists and antagonists have been shown to affect attention to time in rats in a study using a peak interval procedure, where the signal being timed was interrupted and the animals had to adjust to stopping and restarting their internal timing mechanism (Buhusi and Meck 2002).

Clinical Research

The genesis of PD is the depletion of DA producing neurones in the SNc, leading to an imbalance in the excitatory and inhibitory activity in the direct and indirect pathways within the basal ganglia. In PD, DA depletion is greatest in the putamen, the primary basal ganglia component in the frontostriatal motor loop. This circuit connects the basal ganglia to the motor cortex, the supplementary motor area (SMA), and the lateral premotor cortex and has a primary role in movement (Alexander et al. 1986). Typically, patients present with increased muscle tone (rigidity/muscle stiffness), bradykinesia (slowness of movement), akinesia (poverty or absence of movement), tremor (4–5 per second at rest), and balance and walking problems (a shuffling gait). The bradykinesia and motor slowness have been at the forefront of shaping the hypothesis that the basal ganglia play a role in temporal processing. Further, assessment of timing behavior both "on" and "off" dopaminergic medication, which alters the efficacy of basal ganglia function in PD, has proved to be an additional tool in investigating the contribution of the basal ganglia and its DA activity to temporal processing. Implanted electrodes for the purposes of deep brain stimulation (DBS) therapy have provided another mechanism for exploring basal ganglia function in PD. To this end, recordings from the internal segment of the globus pallidus in two patients with PD showed that the amplitude of oscillatory activity at around 25 Hz correlated with task duration in a simple timed movement task (Brown et al. 2002).

Many studies have used the Wing and Kristofferson (1973a,b) paradigm and model of repetitive tapping to study motor timing. In this task, during the "synchronisation phase" the participant taps (usually using the right index finger) in synchrony with a series of tones presented at regular intervals, this enables the frequency to become entrained. After the criterion number of taps, the pacing stimulus is switched off and the participant attempts to maintain the frequency unaided in the "continuation phase". Wing and Kristofferson's model breaks down the

variance of the inter-response interval during the continuation phase into variance associated with a central “clock” and variance associated with motor implementation. Accuracy, i.e., the mean inter-response interval, is an additional metric of motor timing proficiency. The results using this task are varied, with most studies reporting a deficit in patients with PD (e.g. Harrington et al. 1998; Merchant et al. 2008; O’Boyle et al. 1996; Pastor et al. 1992a), but with some contradictory evidence (e.g. Ivry and Keele 1989; Spencer and Ivry 2005). Variability tends to be higher in PD compared with controls (e.g. Harrington et al. 1998; Merchant et al. 2008; Pastor et al. 1992a), and also worse “off” medication than when patients are medicated with levodopa (Merchant et al. 2008; O’Boyle et al., 1996). Further, clock variability seems more affected than the motor variability (Harrington et al. 1998; O’Boyle et al., 1996), which is important to the assertion that the basal ganglia are important to the calculation of temporal intervals, and not just in motor activity per se. In line with this, a slope analysis technique also showed that increased variability in PD patients “off” medication during motor timing was specific to time-dependent processes (Merchant et al. 2008). Finally, the inter-response interval, reflecting accuracy, tends to be faster in PD patients than controls when they are tested “on” medication (Harrington et al. 1998; Ivry and Keele 1989; O’Boyle et al. 1996), with a less consistent picture for tapping when “off” medication (O’Boyle et al. 1996; Pastor et al. 1992a). This effect observed “on” medication may reflect the difficulties patients with PD have entraining their “clock” process to the pacing cue during the synchronisation phase (O’Boyle et al. 1996).

The pervasive motor difficulties in patients with PD are a significant caveat when interpreting motor timing impairments, although the parcelling of “clock” variability as well as evidence that repetitive tapping proficiency is separable from clinical assessment of motor proficiency (Merchant et al. 2008) is encouraging. However, these patients are not just impaired on timing tasks where a motor component dominates. Other tasks include: (1) time estimation, where the participant is presented with a temporal interval (e.g. marked with an onset and offset stimulus) and is asked to estimate its duration; (2) time reproduction, where the participant has to reproduce a target interval that has previously been presented to them; (3) time production, where the participant produces a time interval without prior exposure to the target interval; (4) duration discrimination, the participant listens to two intervals and has to decide whether the second is longer or shorter than the first, or alternatively whether the second is the same or different than the first. These tasks are usually categorized as perceptual timing tasks as any motor component is minimal and solely used to communicate the participant’s perceptual temporal

judgement, rather than be intrinsically tied with the metering of time per se. With these perceptual timing tasks, PD patients tested “off” medication show a pattern of deficits consistent with a slowed “internal clock”, underestimating the length of temporal intervals and overestimating when reproducing previously presented intervals (Lange et al. 1995; Pastor et al. 1992b). Dopaminergic medication has the effect of improving performance. Similarly, abnormal time reproduction is improved during the stimulation of the subthalamic nucleus in individuals who have had DBS surgery (Koch et al. 2004). However, participants are often instructed to estimate intervals using internal counting at a rate of 1 count per second (Lange et al. 1995; Pastor et al. 1992b, 2005), which introduces a timed motor element that can confound interpretation. In addition, the psychophysical properties of chronometric counting and interval timing are very different, with the variance in chronometric counting not conforming to the scalar property as in interval timing (Hinton and Rao 2004). Although chronometric counting may still utilize internal timing processes (e.g. to generate individual counts), it is a less pure measure of internal timing processes and results in more precise estimations (Hinton et al. 2004). In other studies, random numbers are read aloud during the timing process to inhibit counting (e.g. Koch et al. 2004; Malapani et al. 1998; Riesen and Schnider 2001), but this creates a “dual task” paradigm, which is differentially more demanding for the PD patients (e.g. Brown and Marsden 1991; Perbal et al. 2005). To overcome these methodological problems, we tested time production in the 30–120 s range in individuals with PD in a design that involved no additional cognitive demands and found evidence of an atypical accuracy profile in these patients compared with controls (Jones et al. 2008).

Using very short intervals (<2 s) is another obvious approach to reducing additional cognitive demands. Within this range, deficits in time reproduction, temporal bisection (a human analog of the animal task), and duration discrimination have been recorded in PD (Harrington et al. 1998; Jones et al. 2008; Merchant et al. 2008). Of note, Harrington et al. (1998) reported significant impairment on a duration discrimination task with preserved performance on a frequency discrimination task in PD, suggesting that nontemporal factors were unlikely to underpin the deficit. In a similar vein, Merchant et al. (2008) and Jones et al. (2008) found no association between performance on millisecond-range perceptual timing tasks and measures of memory and attention in their samples of PD patients. However, not all studies of this nature conclude that there is deficit in milliseconds-range temporal processing in PD (Ivry and Keele 1989; Koch et al. 2008; Smith et al. 2007). In fact, Smith and colleagues found evidence of a deficit in temporal sensitivity for seconds-range (1–5 s) temporal bisection but not millisecond-range (100–500 ms) temporal

bisection, although the participants were tested “on” medication only. It is not completely clear whether a temporal deficit in PD is confined to durations within a particular range, notably whether in the milliseconds or seconds range. Although there is evidence of dysfunction at both interval ranges, there may be different, or additional, reasons for attenuated performance in the two ranges. Unlike intervals in the milliseconds range, an association between short term and working memory processes and temporal processing has been shown for intervals in the seconds range (5–120 s) (Jones et al. 2008; Perbal et al. 2005). Although the genesis of PD lies in DA deficiency in the SNc, the depleted DA levels have wide-reaching effects. The fronto-striatal motor loop is the most affected circuit, but as the disease progresses, the impact on the frontal cortex becomes more widespread and cognitive deficits emerge. Thus, it is not inconceivable that deficits in the more cognitively demanding temporal tasks may have a more cognitive origin or influence.

The peak interval procedure has been adapted for use in humans and tested in patients with PD. This task, along with the time reproduction task, has demonstrated an interesting “migration effect” in individuals with PD. It has been observed that shorter intervals (<10 s) are overestimated and longer intervals (≥ 15 s) are underestimated, i.e., these intervals “migrate” together, when presented in consecutive blocks (Koch et al. 2005; Malapani et al. 2002, 1998). Malapani and colleagues required patients to encode and reproduce intervals under different medication states and the results point to a dysfunction in the retrieval of temporal memories, whereby interference occurs between the two remembered intervals (Malapani et al. 2002). A similar migration effect has been found for shorter intervals of 500 and 2,000 ms, with a significant underestimation of the 2,000 ms interval observed in the patient group (Koch et al. 2008). Koch and colleagues posited a cognitive explanation, such as set-shifting difficulties, as they did not find impairment when the two blocks were separated by 1 h (albeit in a different group of patients). The dissociation between temporal processing deficits and wider cognitive deficits, including general discrimination ability (Harrington et al. 1998) and working memory and attention (Jones et al. 2008), suggests that general cognitive dysfunction does not suffice as an explanation of timing deficits in PD. However, the consideration of the possible interaction between broader cognitive processes and timing function may prove beneficial in future studies.

A recent study by Merchant and colleagues (Merchant et al. 2008) draws attention to a phenomenon that is largely ignored in the temporal processing literature: heterogeneity of temporal processing within PD. The nineteen patients who took part could be statistically divided into those who showed “high” variability within each of three timing tasks

and those who showed “low” variability. Although those with high variability were significantly different from the control group, those with low variability did not differ from controls, even when “off” medication. Heterogeneity might partly explain the mixed results in the literature, particularly given the small sample sizes often used. As PD is a disorder with a well-described etiology, experimental research does not tend to be drawn to exploring individual differences. It is important to consider whether variation in performance can be meaningfully linked to hypothesized biological markers of temporal processing related to the basal ganglia and DA. Or more broadly, if there are timing-related performance differences between clinically identified subgroups (e.g. Lewis et al. 2005; Schrag et al. 2006).

PD is not the only pathology that affects the dopaminergic system and the basal ganglia. To our knowledge, there has only been one study of the timing performance of patients with circumscribed lesions to the basal ganglia. Perhaps surprisingly, these patients showed no impairment on the repetitive tapping task (Aparicio et al. 2005). However, the study had a small sample size ($N=6$) and the patients all had unilateral lesions in the left hemisphere. It is unclear whether bilateral or right-sided lesions would present a different picture. Huntington’s disease is another neurodegenerative disorder that affects the basal ganglia and there is evidence that gene-carriers close to developing the disorder have smaller caudate volumes and poorer duration discrimination ability than healthy controls, whereas those who are further from developing the disorder do not differ from controls (Paulsen et al. 2004). Similarly, timing variability on the repetitive tapping task relates to the proximity of the disease onset (Hinton et al. 2007). The dysfunction of dopaminergic mechanisms has been cited as a principal component of the pathophysiology of schizophrenia (Davis et al. 1991). Measures of time estimation and time production in individuals with schizophrenia have indicated that the patients overestimate time intervals in the 5–60 s range compared with healthy controls, suggesting a slowed “internal clock” (Wahl and Sieg 1980). Studies have also found deficits in patients with schizophrenia in the duration discrimination, temporal bisection, and temporal generalization of millisecond-range intervals (Carroll et al. 2008; Davalos et al. 2003; Elvevag et al. 2003; Rammsayer 1990). Further, these deficits could not be explained by differences in attention or working memory (Elvevag et al. 2003; Rammsayer 1990). Volz et al. (2001) observed impaired duration discrimination, but not impaired frequency discrimination, suggesting a specific temporal deficit in patients with schizophrenia; which was furthermore related to decreased activation in the putamen and associated frontal and thalamic circuitry. Temporal processing deficits have

also been observed in attention-deficit hyperactivity disorder (ADHD), a developmental condition that encompasses overactive behavior (i.e. hyperactivity), impulsive behavior, and difficulty in paying attention and in which the dopaminergic system (see Tripp and Wickens 2008) and basal ganglia (e.g. Max et al. 2002; Semrud-Clikeman et al. 2000; Teicher et al. 2000) have been implicated. Individuals with ADHD are poor at timing tasks including duration discrimination (e.g. Radonovich and Mostofsky 2004; Rubia et al. 2003; Smith et al. 2002; Yang et al. 2007) and time reproduction (e.g. McInerney and Kerns 2003; Mullins et al. 2005), although there is some inconsistency in the evidence (see Toplak et al. 2006 for a review). There is evidence that these temporal processing deficits may relate to the concomitant cognitive difficulties, including attention, working memory, and inhibition (e.g. Kerns et al. 2001; McInerney and Kerns 2003), although there have been some examples of dissociation (e.g. Smith et al. 2002a,b). One major obstacle to the interpretation of the findings in schizophrenia and ADHD is that patients are typically tested when medicated, making it difficult to dissociate the effects of neuropathology from the influence of medication. For example, neuroleptics affect temporal performance in healthy adults (Rammsayer 1993, 1997) as well as modulate cortical and subcortical activity (Muller et al. 2003). One alternative approach in the field of research relating to schizophrenia is to test unaffected individuals with a high genetic risk for developing the disorder. To this end, Penney et al. (2005) observed that performance on a temporal bisection task (3 or 6 s) was impaired in high-risk individuals who did not have a diagnosis of schizophrenia. A further note of caution comes from evidence that both disorders affect cerebellar function (e.g. Andreasen and Pierson 2008; Toplak et al. 2006) and there is evidence to suggest that healthy cerebellar function may be necessary for certain types of temporal processing (e.g. Ivry 1996).

Imaging Research

MRI and PET techniques have provided further insight into the network of regions engaged in temporal processing. Motor timing studies using the repetitive tapping paradigm show activation in the putamen/pallidum and caudate for synchronized tapping (Jantzen et al. 2004; Riecker et al. 2003), continuation tapping, (Lewis et al. 2004; Rao et al. 1997) or both (Jantzen et al. 2004), although one study reports no basal ganglia activation (Jancke et al., 2000). An obvious caveat to these data is that the control task tends to be rest or passive listening, such that disambiguating timing-specific activity from activity related to motor execution is difficult. The duration discrimination

task is perhaps the optimal task for avoiding motor confounds. Of twelve studies that have used this paradigm, either compared with rest, passive listening with cued finger movement, or an alternative discrimination paradigm (e.g. intensity), ten have found evidence of basal ganglia activation, typically in the putamen and caudate nucleus (Coull et al. 2008; Ferrandez et al. 2003; Harrington et al. 2004a; Jueptner et al. 1995; Lewis and Miall 2003a; Livesey et al. 2007; Macar et al. 2006; Maquet et al. 1996; Nenadic et al. 2003; Pouthas et al. 2005; Rao et al. 2001; Tregellas et al. 2006). These activations characteristically appear in concert with prefrontal and motor regions, suggesting the importance of fronto-striatal circuitry. The cerebellum, another region proposed to play a role in temporal processing (e.g. Ivry 1996), was activated in five of the studies. Event-related fMRI methodology has enabled the disambiguation of activation during different stages of the duration discrimination task. The three studies using this protocol all found striatal activation during the encoding/storage phase of the task (Coull et al. 2008; Harrington et al. 2004a; Rao et al. 2001). Rao et al. (2001) found cerebellar activation only at the end of the task, during the decision phase. As such, they suggest the cerebellum could be monitoring and optimizing (auditory) sensory input from the cerebral cortex, which would be important for the comparison of durations held in working memory. Harrington et al. (2004a) found cerebellar activity during the encoding phase, although it should be noted that the study only used rest as a comparison. Further suggestion that the basal ganglia are a crucial component to temporal processing comes from evidence that activity in the striatum is linked to task difficulty and to the accuracy of performance (Coull et al. 2008; Harrington et al. 2004a; Tregellas et al. 2006). The basal ganglia are also activated by time reproduction (Buetti et al. 2008; Hinton and Rao 2004; Jahanshahi et al. 2006), although in one study only a source of basal ganglia output, the supplementary motor area (SMA), was activated (Macar et al. 2002). Basal ganglia activation is also common during time production, but does not survive comparison with a matched task involving force production (Macar et al. 2004; Macar et al. 2006; Lewis and Miall 2002), unlike activation in the SMA and pre-SMA.

Overall, imaging research supports the role of the basal ganglia in temporal processing. Additionally, there is evidence for the activation of other parts of the fronto-striatal motor circuit, particularly the SMA. The basal ganglia activation is almost exclusively within the striatum, despite animal work that has implicated the SN. However, we have recently reported evidence of the activation of the SNc during a time reproduction task, compared with a reaction time task that is closely matched for additional motor and cognitive components (Jahanshahi et al. 2006).

Models of Basal Ganglia Functioning During Temporal Processing

One point of contention in the literature is whether intervals of different length (e.g. milliseconds vs. seconds) are timed using different neural systems. A systematic review of the functional imaging literature suggests a dissociation between regions activated by an “automatic” timing system that computes milliseconds-range intervals that are defined by movement and continuous in nature, and a “cognitively controlled” timing system that is preferentially involved in the measurement of discrete seconds-range intervals that are nonmotor (Lewis and Miall 2003b). “Automatic” type tasks activate the motor system, with the overlearned nature of the tasks removing the need for attentional modulation, whereas “cognitively controlled” type tasks activate prefrontal and parietal areas associated with working memory and attention. This dichotomy does not encapsulate every type of timing (for example, millisecond-range perceptual discrimination of intervals that are not movement-based or continuous), but aligns with previous hypotheses that seek to divide temporal processing into short, subcognitive, and motor-system dominated intervals and long, cognitively-mediated intervals (e.g. Rammsayer 1993, 1997). It has been hypothesized that the basal ganglia are engaged in the timing of seconds-range intervals and that the cerebellum is engaged in the timing of millisecond-range intervals (e.g. Ivry 1996). This hypothesis is particularly appealing, given the established role of the cerebellum in time-dependent conditioned eyeblink responses (e.g. Woodruff-Pak et al. 1996; Yeo et al. 1985a, b) and evidence of millisecond-range timing deficits in patients with lesions to the cerebellum (Casini and Ivry 1999; Ivry and Keele 1989; Ivry et al. 1988; also see Harrington et al. 2004b). Further, coupling more “cognitive” timing with the basal ganglia, which have such intimate links to the frontal cortex, has an intuitive appeal. However, this hypothesis cannot be reconciled with evidence of deficits in the processing of extremely short (<40 ms) and millisecond-range intervals in patients with PD (e.g. Artieda et al. 1992; Harrington et al. 1998; Merchant et al. 2008), with evidence of the effect of DA antagonists on the temporal processing of extremely short durations (50 ms) (Rammsayer 1993, 1997), or with reports of basal ganglia activation during millisecond range temporal processing in imaging studies (e.g. Jahanshahi et al. 2006; Jueptner et al. 1995; Rao et al. 1997). The evidence of the role of the basal ganglia in seconds-range intervals is equally pervasive (e.g. Pastor et al. 1992b; Jahanshahi et al. 2006; Jones et al. 2008).

There is clear evidence for the engagement of the basal ganglia and the critical role of DA in temporal processing. However, the challenge remains to model timing behavior within such a complex structure with inherent

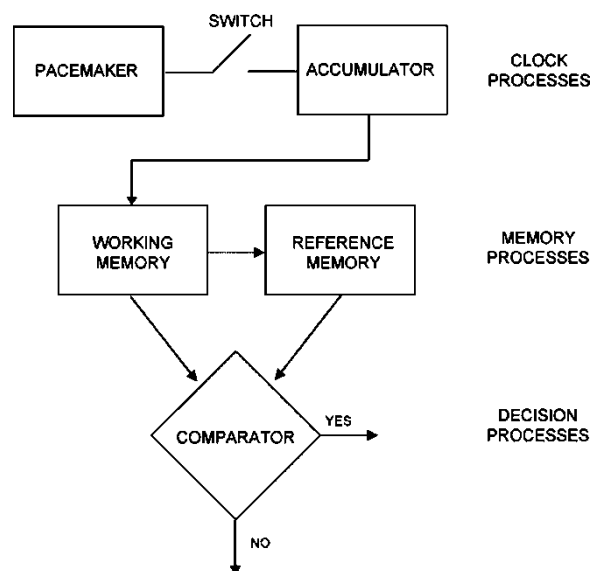


Fig. 1 Schematic representation of the SET model, Gibbon and Church (1984)

neurophysiological constraints. Easily, the most influential model of temporal processing is Scalar Expectancy Theory (SET: Gibbon 1977; Gibbon and Church 1984; Gibbon et al. 1984 and see Wearden 1999 for a more current discussion), an information processing model of interval timing. Briefly, the model conceives three processing stages: clock, memory, and decision (see Fig. 1). The clock stage consists of a pacemaker that emits pulses, with the pacemaker being connected to an accumulator via a switch. At the onset and offset of an interval that is to be timed, the switch is operated by a timing signal and the pulses are gated from the pacemaker to the accumulator. The accumulator therefore holds the current time value, which can be “downloaded” to a working memory store. Important time values, e.g., those associated with reinforcement in animal studies or a standard interval in a human study, are transferred from working memory to reference memory, a more permanent store. The decision to respond occurs in the comparator, where the current time in working memory is compared with the stored time in reference memory. The SET model subscribes to the observation that interval timing in humans and animals conforms to the “scalar property”, whereby the standard deviation of the response distribution increases with the mean of the interval being timed. Animal, clinical, and imaging work have concurred with the hypothesis that the basal ganglia and DA form the pacemaker-accumulator, i.e., the “clock” component, of the SET model (e.g. Matell and Meck 2004; Meck 1996). Specifically, it has been suggested that neurons within the SNc form the pacemaker, with the rate of the pacemaker being modulated by the effective level of DA and the integration of the pacemaker pulses (i.e. neuronal firing) occurring in the striatum (see Matell

and Meck 2004). Further, memory and decision processes have been conceived as residing in the frontal cortex (e.g. Meck 1996). However, it should be noted that work with patients with PD suggests the influence of DA on the encoding and retrieval of temporal memories (Malapani et al. 1998, 2002). This finding is problematic for the neurobiological interpretation of SET, which conceives that dopaminergic activity is mediating clock function. Perhaps most problematically, it is unclear that the physiology of the basal ganglia could support an accumulation process lasting minutes (see Matell and Meck 2004).

SET is a pacemaker-accumulator model, an alternative approach are oscillatory models, which conceive that time is distributed along a series of neural oscillators (e.g. Miall (1989, 1992, 1996)). Matell and Meck (2000) argue that oscillatory models are the most biologically plausible of the attempts to capture temporal processing within a theoretical framework. As a result, the striatal beat frequency model (SBF: Matell and Meck 2000, 2004) adapts an early neural network model of oscillatory activity (Miall 1989 and 1992) to fit the neurophysiological constraints of the cortico-striatal-thalamic loop. Essentially, it is proposed that the detection of coincident neural activity, known to be a function of the striatum, encodes temporal durations. Cortical and thalamic input to the striatum serves as the oscillatory activity (i.e. clock signal), whereas striatal spiny neurons act as “coincidence detectors” firing when a set of oscillating neurons oscillate with the same beat frequency (i.e. integrating the clock signal to produce a “decision”). The beat frequency is the lowest common multiple of the periods of the different oscillations that are in phase with the length of the interval, i.e., it defines the temporal duration. Dopaminergic input from the SNc is posited as a reinforcement signal for important durations, with the dopaminergic activity strengthening the cortico-striatal synaptic weights that consequently code durations (i.e. memory storage). Dopamine within the SNc also resynchronizes cortical oscillators and resets the coincidence detection neurons at the onset of a stimulus to be timed, acting as a “perceptual starting gun” (Matell and Meck 2000). If the SNc has a principal role in initiating the timing process, then this may explain why it is not often significantly active during imaging studies, where data are typically pooled across the whole temporal task. The study that did find SNc activation (Jahanshahi et al. 2008) used a control task that tightly matched for nontemporal motor and cognitive components and involved time reproduction, for which responding efficiently to a “go” signal is essential. Matell and Meck (2004) describe various weaknesses in the model, including whether such long and precise oscillations are possible in the cortex and the whether the model has the capacity to account for complex temporal conditions such as simultaneous temporal processing. Coull et al. (2008) did not find evidence of

striatal activation during the retrieval/comparison stage of a duration discrimination task. They discuss that the SBF model would predict striatal activation at this stage, due to the proposed role in detecting coincident neural activity. However, the activation of the putamen has been found during the decision stage in a separate study of duration discrimination (Harrington et al. 2004a). Importantly, the SBF model demonstrates the scalar property.

It should be noted that there are other models of interval timing, which have received less attention from those interested in mapping behavior onto neural substrates. These include the behavioral theory of timing (Killeen and Fetterman 1988), which concurs with SET in proposing that a pacemaker and accumulator underpin timing. However, in contrast to the more cognitive approach, this theory considers behavior to be the mediator of temporal judgements. It is hypothesized that animals meter time by moving through a series of behavioral states (e.g. running to the back of the cage, sitting, scratching a leg, and so on) and that this series of behavioral states can become reinforced and act as conditional stimuli. The pacemaker is presented as a biological oscillator and each pulse that is registered moves the animal onto the next behavioral state, a transfer that occurs with a constant probability. From a different perspective, Staddon and Higa's (1999) multiple time scale model suggests that temporal information is derived from memory data and depends on the same mechanism as habituation. Temporal judgements are based on memories of different “strengths”, i.e., a memory decays as time passes and this change is quantified in a systematic, predictable way by the organism.

Concluding Remarks

The consensus of evidence from animal, pharmacological, clinical, and imaging work all point to the importance of the basal ganglia and dopamine in milliseconds and seconds-range temporal processing at both the perceptual and motor level. The basal ganglia do not work in isolation and striatal-thalamo-cortical connections are likely involved as part of a network of regions that meter this behavior. Attempts to decipher the specific roles of the basal ganglia have produced varied results, partly because of the methodological limitations of various techniques. Particularly, evidence for the role of the SN in temporal processing is far more compelling in animal lesion work than it has been in human imaging work, perhaps partly because of the limited spatial resolution of imaging techniques, albeit that is gradually improving, and partly due to the design limitations and a failure to use adequate control tasks to control motor

elements and tease out timing-specific patterns of neural activation. Only one model, the striatal beat frequency model, has attempted to align the neurophysiological complexities of the basal ganglia with temporal processing mechanisms in a biologically plausible way.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 14

Electrophysiological and Neurochemical Characterization of 7-Nitroindazole and Molsidomine Acute and Sub-Chronic Administration Effects in the Dopaminergic Nigrostrial System in Rats

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Abstract Nitric oxide (NO) plays an important role in the integration of information processed by the basal ganglia nuclei. Accordingly, considerable evidence has emerged indicating a role for NO in pathophysiological conditions such as Parkinson's disease (PD) and other neurodegenerative disorders. Despite these recent advances, the nitrgic modulation of the dopamine (DA) nigrostriatal system is still unclear. In order to fill this gap, in this study we used in vivo electrophysiology and ex vivo neurochemical analysis to further investigate the effect of NO signaling in rat substantia nigra pars compacta (SNc) and the striatum. Acute and subchronic (4 days) pharmacological manipulation of the NO system using 7-nitroindazole (7-NI, 50 mg kg⁻¹ i.p.) and molsidomine (MOL, 40 mg kg⁻¹ i.p.) treatment caused significant changes in both DA SNc neurons electrophysiological properties and striatal DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels. It is worth noting that acute inhibition of NO production decreased DA nigrostriatal neurotransmission while its subchronic inhibition was instead excitatory. Thus, a crucial role for NO in the modulation of nigrostriatal DA function is suggested together with a potential role for inhibitors of NO sythase in the treatment of PD.

Keywords Cells-per-track • Dopamine • Extracellular recording • HPLC • Nitric oxide • Parkinson's disease

Abbreviations

6-OHDA	6-hydroxydopamine
7-NI	7-nitroindazole
ACh	Acetylcholine

BBB	Blood brain barrier
DA	Dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
l-ARG	l-Arginine
l-NAME	N-ω-nitro-l-arginine methyl ester
l-NOARG	l-nitro-arginine
MAO	Monoamine oxidase
MOL	Molsidomine
NNLA	N-nitro-l-arginine
nNOS	Neuronal NO synthase
NO	Nitric oxide
oPFC	Orbital prefrontal cortex
PD	Parkinson's disease
POPAC	Dihydroxyphenilacetic acid
PPT	Pedunculopontine tegmental nucleus
SIN-1	3-morpholinosydnnonimine
SNc	Substantia nigra pars compacta
VTA	Ventral tegmental area

Introduction

Nitric oxide (NO) is a highly reactive gaseous molecule almost ubiquitous in the human body (Bian and Murad 2003). It plays a fundamental role in the modulation of a large range of physiological processes, from erectile function (Feifer and Carrier 2008) to cognition (Thatcher et al. 2006). Compelling evidence has shown that NO controls motor behavior modulating the integration of information processed by the basal ganglia nuclei. Most likely, it interacts with dopaminergic (DAergic), serotonergic, cholinergic, and glutamatergic neurotransmission at different levels of these nuclei (West et al. 2002; Del Bel et al. 2007). The presence of this fine modulation is supported by anatomical evidence showing the presence of nitrgic neurons throughout all the basal ganglia nuclei including the substantia nigra pars compacta (SNc) and in other regions involved in motor control such as the motor cortices and the pedunculopontine tegmental nucleus (PPT), although their number differs

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significantly in these areas (Bredt et al. 1990; Vincent and Kimura 1992; Egberongbe et al. 1994; Nisbet et al. 1994; Garthwaite and Boulton 1995; Eve et al. 1998; Leontovich et al. 2004; Del Bel et al. 2007). Despite the large body of experimental evidence, the exact role played by NO in motor control is far from completely understood. Indeed, in vivo studies using extracellular recordings combined with local or systemic drug administration have shown results that are sometimes controversial and difficult to integrate with the function model of the basal ganglia (West and Grace 2000; West et al. 2002; Sardo et al. 2002; Di Giovanni et al. 2003, 2006; Liu et al. 2005; Sardo et al. 2003, 2006; Galati et al. 2008). Furthermore, contrasting results also exist regarding the NO modulation of striatal DA release (see West et al. 2002).

Conversely, compelling animal and human evidence indicates a causative role for NO in the DA neuronal death seen in Parkinson's disease (PD) (Di Matteo et al. 2006, 2009; Del Bel et al. 2007; Di Giovanni 2007; Esposito et al. 2007; Gomes et al. 2008). Consistently, mice mutant for neuronal NO synthase (nNOS) showed altered locomotor abilities, and rats and mice treated with various NOS inhibitors had problems with fine motor control. NO, furthermore, antagonized the increase in locomotor activity found after the administration of DA agonists. In addition, the pharmacological blockade of nNOS decreases locomotion and induces catalepsy in different animal species (see Del Bel et al. 2005, 2007 for comprehensive reviews).

On the basis of these considerations, a better understanding of the NO modulation of the nigrostriatal DA system appears to be of paramount importance. For this purpose, in this study we used standard in vivo extracellular recording technique and cells-per-track protocol to study SNc DA neuronal activity, and ex vivo neurochemical analysis for the evaluation of striatal levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid levels (DOPAC) during the pharmacological manipulation of NO system. We employed 7-nitroindazole (7-NI), a relatively specific neuronal inhibitor of nNOS, and molsidomine (MOL), a NO donor. Moreover, to evaluate eventual plastic changes of NO system, we also examined the effect of 7-NI and MOL subchronically administered (4 days). Our results may contribute to a better understanding of basal ganglia physiology and their disorder conditions such as PD.

Materials and Methods

Male Sprague–Dawley rats, from Charles River Laboratories (Calco, Varese, Italy) were housed at appropriate environmental conditions ($21 \pm 2^\circ\text{C}$ room temperature, 12-h light/dark cycle, and 40–60% humidity). Water and food were

provided ad libitum. Procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992) and international laws and policies (*EEC Council Directive* 86/609, OJ L 358,1, Dec. 12, 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication N. 85-23, 1985; and *Guidelines for the Use of Animals in Biomedical Research*, *Thromb. Haemost.* 58, 1078-1084, 1987).

Electrophysiological Recording

Experiments were performed in chloral hydrate-anesthetized rats (400 mg kg^{-1} , i.p.; with supplemental doses administered via the lateral tail vein). Throughout the experiment, body temperature was maintained at $36\text{--}38^\circ\text{C}$ with a heating pad.

Nigral DA cells were recorded extracellularly as described previously (Bunney et al. 1973; Shi et al. 1997). Single-cell recording procedures were performed by using single micropipettes. The single micropipettes were filled with a 2.0 M NaCl solution containing 2% Pontamine Sky Blue dye. The tip of the electrode was broken back under a microscope until a diameter of between 1 and $2 \mu\text{m}$ was obtained. The impedance of the electrode was measured between 5 and $15 \text{ M}\Omega$. A small burr hole was drilled above the SNc (3.0 mm anterior to the lambdoid suture and 2.0 mm lateral to the midline, according to Paxinos and Watson 1986). DA cells were found between 6.5 and 8.5 mm below the cortical surface. Electrical signals of spike activity were passed through a high-impedance amplifier the output of which was led into an analog oscilloscope, audio monitor, and window discriminator. Unit activity was then converted to an integrated histogram by a rate-averaging computer and displayed as spikes per 10-s intervals.

The cells-per-track experiments were performed blind with regard to the treatment condition of the animal. 60 min after the drug treatments, single spontaneously firing dopaminergic cells were counted and recorded for 5 min by lowering the electrode through a block of tissue ($240,000 \mu\text{m}^2$) within the SNc, which could be reproducibly located from animal to animal (Shim et al. 1996). Twelve electrode tracks (separated from each other by $200 \mu\text{m}$), the sequence of which was kept constant from animal to animal, were carried out.

Neurochemical Assay

To establish the DA and DOPAC levels in the striatum, rats were sacrificed by cervical dislocation. The brains were

rapidly removed and immediately placed into ice-cold saline. The right and left striata were then dissected on an ice-cooled plastic dish and stored at -80°C , until biochemical assay.

For HPLC analysis, tissue samples were weighed, transferred into 1 ml 0.1 N perchloric acid (HClO_4) and $0.45\text{ }\mu\text{M}$ of 3,4-dihydroxybenzylamine (DHBA), and afterward homogenized for 1 min by ultrasounds and subsequently centrifuged for 15 min at 12,000 rotations per min and 4°C . The centrifugate was filtered through a membrane filter with a pore size of $0.45\text{ }\mu\text{m}$ before HPLC assay.

Dialysate samples were analyzed by reversed-phase HPLC coupled with electrochemical detection. The mobile phase was composed of 70 mM NaH_2PO_4 , 0.1 mM Na_2EDTA , 0.7 mM triethylamine, 0.1 mM octylsulfonic acid, and 10% methanol, adjusted to pH 4.8 with orthophosphoric acid. This mobile phase was delivered at 1 ml min^{-1} flow rate (LC-10 ADvp pump, Shimadzu Italia, Milano) through a SupelcosilTM column (LC-C8, $4.0 \times 250\text{ mm}$, $5\text{ }\mu\text{m}$, Supelco, Bellefonte, PA, USA). Samples were injected manually into the HPLC and the detection of DA and DOPAC was carried out with a coulometric detector (Coulchem II, ESA, Bedford, MA, USA) coupled to a dual electrode analytic cell (model 5014). The potential of the first electrode was set at 0 mV and the second at +400 mV. Under these conditions, the sensitivity to DA was $0.35\text{ pg}/20\text{ }\mu\text{l}$ with a signal-to-noise ratio of 3:1.

Study Design

For the extracellular recordings, 7-NI was dissolved in 50% dimethyl sulfoxide (DMSO), while MOL was dissolved in saline. The drugs were administered i.p. in a volume of 10 ml kg^{-1} . For the acute studies, animals received a single dose of 7-NI (50 mg kg^{-1}) or MOL (40 mg kg^{-1}) intraperitoneally administrated. In the subchronic experiments, rats were treated for 4 days once a day with 7-NI (50 mg kg^{-1} , i.p.) or MOL (40 mg kg^{-1} , i.p.). On the fifth day, they again received 7-NI (50 mg kg^{-1} , i.p.) or MOL (40 mg kg^{-1} , i.p.) injection. Control rats were injected with an equal volume of vehicles only. Cells-per-track experiments and tissue samples collections were carried out an hour after the last drug treatments.

Histology

To allow the histological examination of the recording site, a spot of dye was injected at the end of the recording by passing a $-30\text{ }\mu\text{A}$ current through the electrode for 15–20 min. Animals were deeply anesthetized with chloral hydrate

and perfused through the heart with physiological saline followed by 10% formalin. Brains were removed and placed in 10% buffered formalin for two days before histological examination. Frozen sections were cut at $40\text{-}\mu\text{m}$ intervals and stained with thionin. The microscopic examination of the sections was carried out to verify that the electrode tip was in the SNc.

Statistics

For the electrophysiological recordings, the statistical significance of the effect of the drug treatments was determined by comparing the number of dopaminergic neurons counted per track, their firing rates, and percentage of spike fired in bursts drug treatments. All numerical data were expressed as mean $\pm$ SEM. Differences between groups were evaluated by using analysis of variance (ANOVA) followed by post-hoc Tukey HSD tests.

For the neurochemical data, quantification was performed by the internal standard method (DHBA) based on the area under the peak. Data corresponding to mean $\pm$ SEM values of absolute DA and DOPAC levels, obtained in each experimental group, were normalized by tissue weight (ng/g) and analyzed by ANOVA.

All statistical analyses were performed with StatViewTM version 5.0.1 (SAS Institute Inc., Cary, NC, USA).

Drugs

7-NI and MOL were purchased from Sigma, Milano, Italy. Chemicals used for HPLC analysis, DHBA, and perchloric acid were purchased from Carlo Erba, Milano, Italy.

Results

Effect of acute and subchronic administration of 7-nitroindazole and molsidomine on the dopamine neuron population activity in the substantia nigra pars compacta

To test whether the pharmacological manipulation of NO system may influence the population activity of SNc neurons, a series of cells-per-track experiments were carried out. The effect of the vehicle of 7-NI was not different from the vehicle of MOL (saline) in both acute and subchronic treatments; therefore, these data were pooled together and are subsequently referred to as the control vehicle. Rats that

received control vehicle infusions ($n=10$) exhibited an average of 1.08 ± 0.1 spontaneously active dopamine neurons per electrode track (a standard measure of population activity), which fired at an average rate of 4.0 ± 0.2 Hz with $22.7 \pm 2.3\%$ of action potentials fired in bursts, consistent with previous findings (Shim et al. 1996) (Fig. 1a–c, white bars). A single injection of 7-NI (50 mg kg^{-1} , i.p.; $n=6$) produced a significant ($\approx 30\%$, $P < 0.05$) decrease in the bursting activity of dopamine neurons relative to control treatments while having no effect on the number of spontaneously active dopamine neurons or mean firing rate of these cells (Fig. 1a–c, black bars). Repeated i.p. treatment with 7-NI (50 mg kg^{-1}) for

four consecutive days caused a more significant reduction ($\approx 65\%$, $P < 0.01$) in the burst firing (Fig. 2a–c) and in contrast increased the number of spontaneously active dopaminergic neurons ($\approx 45\%$, $P < 0.01$), while still having no effect on their mean firing rate (Fig. 1a–c, diagonal line bars). On the other hand, only acute MOL (50 mg kg^{-1} , i.p.) treatment was capable of increasing the number of spontaneously active DA neurons by 30% ($P < 0.05$), while its repeated administration showed no effect (Fig. 1a, gray bars). Moreover, both acute and subchronic treatment did not significantly alter the mean firing rate and percentage of spikes fired in bursts (Fig. 1b, c gray and horizontal bars).

Fig. 1 Effect of acute and sub-chronic administration of 7-nitroindazole and molsidomine on the dopamine neuron population activity in the substantia nigra pars compacta. (a) The mean number of active DA cells in the SNc was significantly increased either by repeated (4 days) intraperitoneal administration of 7-nitroindazole (7-NI, 50 mg kg^{-1} ; $n=5$; diagonal line bar) or acute treatment with molsidomine (MOL, 40 mg kg^{-1} ; $n=5$; grey bar) compared to vehicle treated rats ($n=10$). (b) The average firing rate was not modified by all treatments. (c) A reduction of the percentage of spikes fired in bursts was observed after acute and sub-chronic 7-NI treatments (black diagonal line bars). Each value represents the mean ($\pm$ SEM), (* $P < 0.05$, ** $P < 0.01$ compared with the control group, one-way ANOVA, followed by Tukey's test)

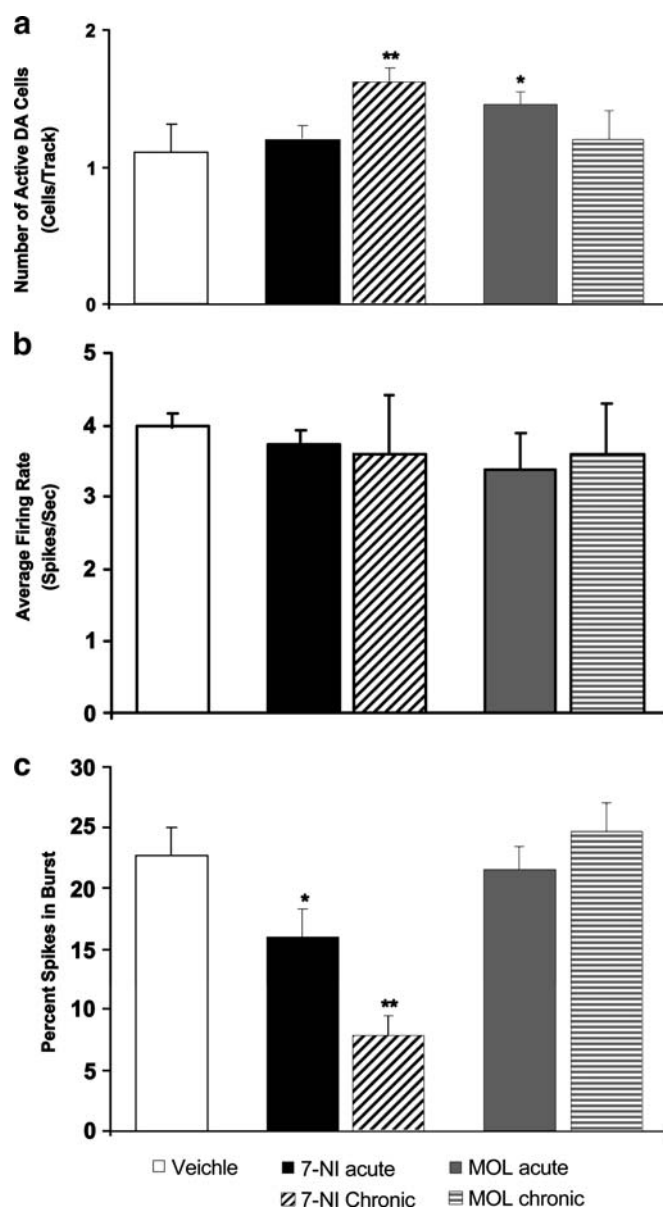
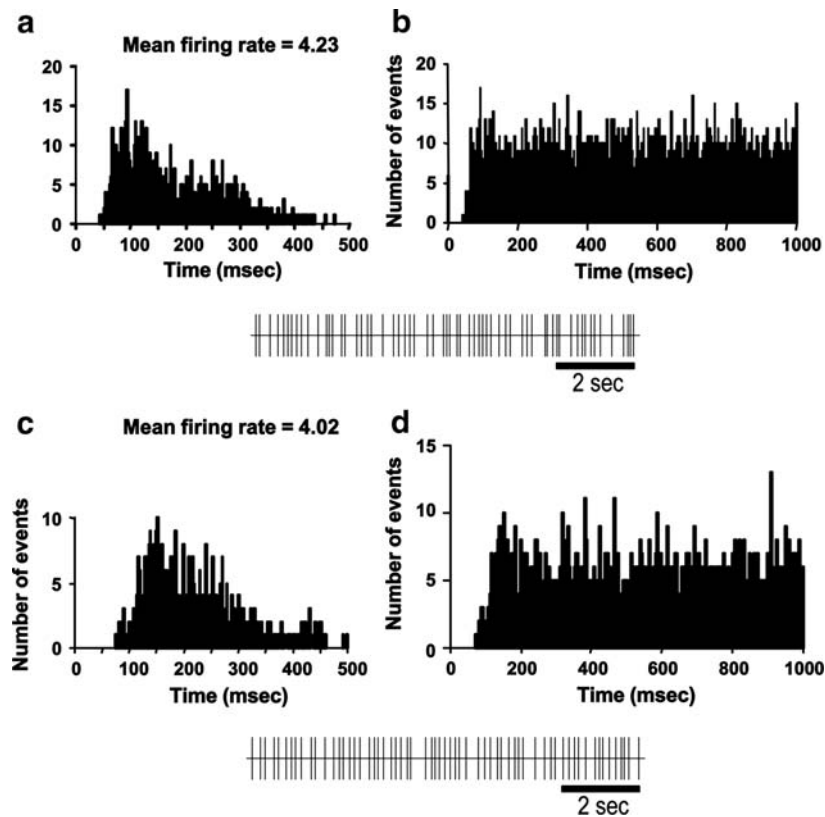


Fig. 2 Effect of vehicle and sub-chronic (4 days) i.p. administration of 7-NI (50 mg kg^{-1}) on the firing rate of DAergic SNc neurons. Representative interspike histograms (ISIH) and autocorrelograms showing the effects elicited by the vehicle (**a, b**) and 7-NI (50 mg kg^{-1} , 4 days) (**c, d**). Below each ISIH and autocorrelogram is the first approximately 10 s of the spike train used to create the autocorrelogram. Bin width = 1 ms



Effect of acute and subchronic administration of 7-nitroindazole and molsidomine on striatal dopamine and dihydroxyphenylacetic acid levels

Results consistent with the cells-per-track experiments were obtained by neurochemical approach. The effect of the vehicle of 7-NI was not different from the vehicle of MOL (saline) in both acute and subchronic treatments; therefore, these data were pooled together and are subsequently referred to as the control vehicle. Acute 7-NI (50 mg kg^{-1} , i.p.; $n = 5$) treatment did not modify significantly the DA levels in the striatum; although DOPAC concentration was drastically reduced by the 7-NI treatment ($-83.1 \pm 4.8\%$, $p < 0.01$) (Fig. 3a, b black bars). Conversely, repeated administration of 7-NI induced a slight increase ($\approx 10\%$) of DA levels and decreased DOPAC levels by $\approx 50\%$ ($p < 0.01$) (Fig. 3a, b diagonal line bars) in the striatum. Furthermore, acute administration of MOL (40 mg kg^{-1} , i.p.; $n = 5$) decreased both striatal DA and DOPAC tissue levels by 30% ($p < 0.05$) and 20% respectively (Fig. 3a, b gray bars). When MOL (40 mg kg^{-1} , i.p.; $n = 5$) was injected subchronically a similar inhibitory trend was revealed, although the modification of both DA and DOPAC levels was not significant (Fig. 3a, b gray and horizontal bars).

Discussion

This study shows for the first time that acute and subchronic pharmacological manipulation of NO system produces complex modifications of DA nigrostriatal transmission in rats as studied with in vivo electrophysiological and ex vivo neurochemical approach. Our findings showed that NO influences DA function in the basal ganglia further supporting its important role in the controlling of motor behavior (see Del Bel et al. 2005, 2007). Here we reported the first in vivo demonstration of NO involvement in the modulation of SNc DA neuron population activity under normal conditions. Indeed, although there have been numerous neurochemical studies that investigated NO modulation of striatal DA release (see West et al. 2002), the effect of NO on nigral neuronal discharge has not been completely clarified yet. The only in vivo study available to date investigated the striatal NO tone modification on DA activity (West and Grace 2000). These authors showed that the increase of NO tone in the striatum was able to counteract the decrease in the firing rate of DA cells observed in control animals during intermittent striatal and orbital prefrontal cortex (oPFC) stimulation as measured with cells-per-track approach. Conversely, the other cell population parameters, i.e., the number of spontaneously active neurons and burst firing, were not modified by the same treatments. On the other hand, the removal of

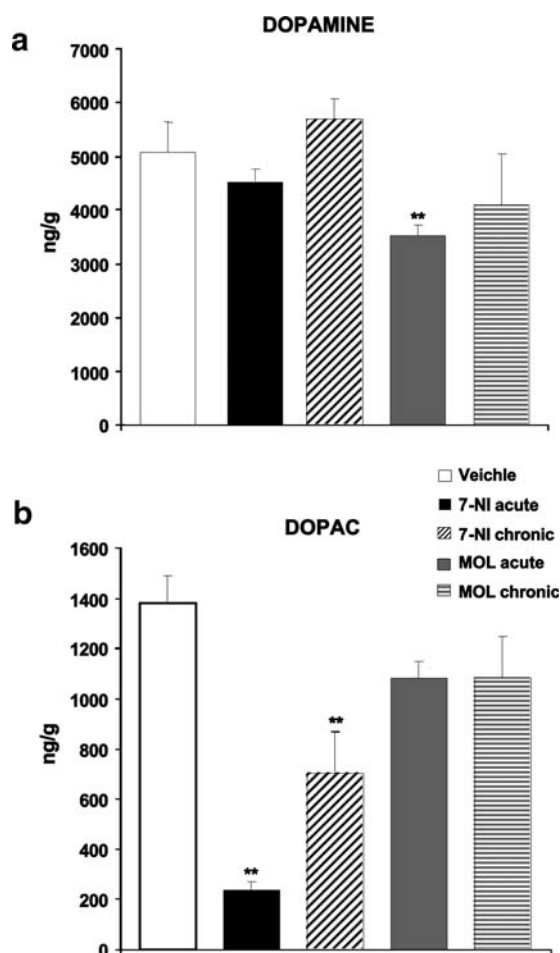


Fig. 3 Effect of acute and sub-chronic administration of 7-nitroindazole and molsidomine on DA and DOPAC on extracellular levels in the corpus striatum. **(a)** The striatal DA levels were lightly increased by repeated (4 days) intraperitoneal administration of 7-nitroindazole (7-NI, 50 mg kg⁻¹; *n* = 5; diagonal line bar) and decreased by acute treatment with molsidomine (MOL, 40 mg kg⁻¹; *n* = 5; grey bar). **(b)** The striatal DOPAC levels were significantly reduced by either acute or sub-chronic 7-NI (50 mg kg⁻¹; *n* = 5) treatments (black and diagonal line bars). Each column shows mean $\pm$ SEM values of DA and DOPAC contents (ng/g) in the striata. Each value represents the mean ($\pm$ SEM), (** *P* < 0.01 compared with the control group, one-way ANOVA, followed by Tukey's test)

striatal NO tone had no effect on nigral DA population activity. Additionally, NOS inhibition increased the proportion of DA neurons responding to striatal stimulation and increased the prevalence of the initial inhibitory responses (West and Grace 2000). Thus, it has been proposed that NO may play a pivotal role in controlling the delicate homeostatic processes that normally provide stability to the DA-nigral system. Indeed, NO may be capable of dynamically regulating the relative phasic DA responsivity via its action on tonic DA levels, in a manner dependent on the arousal state of the animal (West and Grace 2000; West et al. 2002).

Here we reported the effects of acute and subchronic pharmacological manipulation of the nitroergic system by general administration of 7-NI and MOL on the electrophysiological properties of DA SNc neurons and striatal levels of DA and DOPAC. We choose MOL, among NO donors, because it can be administrated peripherally. Indeed, liver esterases convert it to the active metabolite, 3-morpholinosydnonimine (SIN-1), which then releases NO (Nitz and Fiedler 1987; Rosenkranz et al. 1996). In addition, MOL is easily adsorbed, has a high bioavailability, a long-lasting duration of action (Boger et al. 1994) and is likely to cross the blood–brain barrier (BBB) (Maccario et al. 1997; Rigamonti et al. 2001). As far as NOS inhibition is concerned, we used 7-NI, since it offers the possibility of a relatively specific inhibitor of neuronal NOS with no effect on blood pressure (Moore et al. 1993; Southan and Szabo 1996).

Acute inhibition of NO levels by 7-NI treatment decreased the percentage of action potentials fired in bursts, but it did not modify the number of spontaneously active nigral DA neurons observed per electrode track and only caused a nonsignificant decrease in the mean firing rate of these cells. These findings are consistent with the evidence suggesting that NO facilitates, and may be necessary for, the expression of burst firing. Indeed, it has been shown that the NOS inhibitor N- ω -nitro-L-arginine methyl ester (L-NAME) and the NO substrate L-Arginine (L-ARG) treatment modified neither the firing rate of the ventral tegmental area (VTA) DA neurons in vivo (Schilström et al. 2004) nor the firing rate of VTA and SNc neurons in vitro (Cox and Johnson 1998; Schilström et al. 2004). Moreover, NOS inhibition did not alter the burst firing of DA neurons but was instead capable of counteracting nicotine- and NMDA-induced burst firing whereas L-ARG potentiated the latter (Cox and Johnson 1998; Schilström et al. 2004). Consistently, we also found that MOL was capable of inducing a significant increase in the number of spontaneously active SNc neurons but did not modify nigral burst firing confirming the L-ARG lack of effect seen in vivo (Schilström et al. 2004) and in vitro (Cox and Johnson 1998). Therefore, NO may be necessary, but not sufficient, for the induction of burst firing.

Our neurochemical data are, furthermore, in line with the electrophysiological recordings, showing that the reduction of the endogenous nitroergic tone by 7-NI treatment does not influence basal striatal DA levels. Indeed, tonic DA release (corresponding to our DA tissue levels) is dependent on the number of spontaneously active DA cells and their average firing rate, whereas burst firing would determine phasic DA changes (Floresco et al. 2003; Grace et al. 2007). The marked reduction in DOPAC ($-83.1 \pm 3.1\%$) induced by 7-NI is likely to be independent of NO inhibition and a consequence of the well-known strong monoamine oxidase (MAO) type B inhibitory activity possessed by 7-NI (Castagnoli et al. 1997; Boireau et al. 2000; Thomas et al. 2008).

On the other hand, increasing NO levels by acute MOL treatment increased the population activity by 30%, without altering the other electrophysiological parameters. Surprisingly, a single MOL injection instead decreased both the striatal DA tissue levels and DA metabolism, although the latter not significantly. This evidence would suggest that NO has an inhibitory effect on striatal dopamine efflux in accordance with some previous studies (Guevara-Guzman et al. 1994; Silva et al. 1995, 2003; Desvignes et al. 1999). Nonetheless, the role of NO in striatal DA release is one of the most controversial in neuroscience and enhancement is the commonly accepted effect (West et al. 2002). However, a reconciliatory hypothesis has been suggested that NO acts to decrease DA release when the biological system is in a state of oxidative stress (Büyükuysal 1997; Trabace and Kendrick 2000). In contrast, under physiological conditions, NO might facilitate DA release. Indeed, we have revealed an exacerbated effect on DA tissue levels in the 6-hydroxyDA (6-OHDA)-model of PD produced by MOL (Di Matteo et al. 2009). 6-OHDA, indeed, induces the degeneration of nigrostriatal DA-containing neurons by producing reactive oxygen species (Simola et al. 2007).

Moreover, the inhibitory effect of MOL on DA levels might be dose related and indeed a recent *ex vivo* neurochemical study showed a lack of effects exerted by exogenous NO produced by MOL on DA nigrostriatal function at a lower dose (4 mg/kg) (Bishnoi et al. 2009). Nevertheless, the coadministration of MOL was capable of attenuating haloperidol-induced orofacial dyskinesia, oxidative damage and change in striatal dopamine levels without having any effects *per se* (Bishnoi et al. 2009). In addition, MOL reduced dose-dependent haloperidol- and NOS inhibitor, N-nitro-L-arginine (NNLA)-induced catalepsy in rats (Krzaścik and Kostowski 1997).

Furthermore, an interesting observation arising from this study was that repeated (4 days) 7-NI and MOL administration affected nigrostriatal neurotransmission differently. Subchronic MOL treatment failed to modify either the number of spontaneously active neurons or other electrophysiological parameters. On the contrary, striatal DA and DOPAC levels were still decreased, although not significantly.

Strikingly, the inhibition of NOS by 7-NI produced an unexpected significant rise in the number of spontaneously active nigral neurons ($\approx 50\%$) and a correlated slight increase of striatal DA tissue levels. Nevertheless, subchronic 7-NI treatment induced an even stronger regularization of the temporal structure of nigral spiking activity reducing burst firing of $\approx 60\%$ compared with the control group. These findings are in line with the evidence that NOS inhibitors induce catalepsy in rodents (Del Bel et al. 1998, 2004) and decrease exploratory behavior in rats (Del Bel et al. 2002), while both effects disappear after only 4 days of

NOS inhibition (Marras et al. 1995; Del Bel et al. 2002, 2005, 2008; Del Bel and Guimarães 2000). Therefore, the increase in DA neurotransmission after 7-NI subchronic treatment showed by our study might be the mechanism involved in the rapid tolerance development after chronic NOS inhibition. The activation of nigrostriatal system after chronic NO inhibition is difficult to explain and it is likely to be a final resultant of different effects. For example, an increase in the number of nitroergic neurons in the striatum, nucleus accumbens, and in the PPT occurred in rodents that developed tolerance to the cataleptic effect of the nonselective NOS inhibitor L-nitro-arginine (L-NOARG) (Del Bel and Guimarães 2000; Del Bel et al. 2008). These plastic changes result in a partial recovery of striatal NO formation (Del Bel et al. 2008) and might be involved in the rise in striatal DA levels that we observed in our study. Moreover, the increase in the number of spontaneously active nigral DA neurons after subchronic 7-NI treatment might be a consequence of the augmented excitatory PPT input to the SNc (Del Bel and Guimarães 2000; Di Giovanni and Shi 2009), likely mediated by acetylcholine (ACh) inasmuch as NOS largely colocalizes with it (Sugaya and McKinney 1994). On the other hand, the decrease in bursting activity seen after subchronic NO inhibition might depend on the reduced nigral NO levels; indeed nitroergic neurons are reduced within the SNc after L-NOARG subchronic treatment (Del Bel and Guimarães 2000). Furthermore, it is possible to rule out the involvement of changes in D₂ receptors in the effects of chronic NOS inhibition in as much as it has been shown in mice and rats that repeated treatment with L-NOARG failed to change striatal D₂ binding (Koylu et al. 2005) and D₂-mRNA expression in the dorsal striatum and the substantia nigra (Del Bel et al. 2008).

The NO modulation of nigrostriatal DA neurotransmission is complex and far from being completely understood. Indeed, NO influences the neuronal activity of the striatum (Sardo et al. 2002; West et al. 2002; Di Giovanni et al. 2003; Liu et al. 2005; Galati et al. 2008; Ondracek et al. 2008), the globus pallidus (Sardo et al. 2003), subthalamic nucleus (Sardo et al. 2006) and the substantia nigra pars reticulata (Di Giovanni et al. 2006). In addition, as striatal NO controls DA concentration, mutually, striatal NO is also under a DAergic influence (Sammur et al. 2006; Sammut et al. 2007). Indeed, both electric and chemical stimulation of the SNc elicited a robust surge in striatal NO efflux (Sammur et al. 2006; Sammut et al. 2007). This release seems to be neuronally dependent being blocked by pretreatment with nNOS inhibitors, and also evoked only by high-frequency stimulation that resembles the natural burst firing of DA SNc neurons. This last piece of evidence indicates that NO efflux occurs only when DA transmission is phasically increased and suggests that information transmitted via the nigrostriatal

pathway during DA cell burst firing may be processed and/or amplified by NOS interneurons (Sammur et al. 2006; Sammur et al. 2007). DA within the striatum could directly modulate NO efflux, exciting NOS interneurons through the activation of DA_{1/5} receptors present on their somas causing an increased release of NO (Sammur et al. 2006). On the other hand, DA modulates striatal NO levels via D₂ receptors in an opposing manner. This inhibitory control seems to be indirect; it is plausible that D₂ receptors are in fact presynaptic on glutamate and ACh fibers impinging on NOS interneurons (Sammur et al. 2007).

In conclusion, our data in *toto* show that nNOS inhibitor 7-NI deeply influences nigrostriatal neurotransmission further supporting the pivotal role of NO in motor behavior. Moreover, subchronic NOS inhibition produced an enhancement of the number of SNc DA neurons firing spontaneously and slightly increased striatal DA tissue levels likely due to plastic changes that resulted in the nitroergic system. Therefore, NO inhibitors could be considered for human use in the treatment of akinesia and rigidity seen in PD and antipsychotic-induced extrapyramidal side effects. The challenge for pharmaceutical research now is to achieve selective inhibition of NOS isoforms; a situation complicated by the possibility that NOS inhibitors can indiscriminately affect beneficial and pathological NO signaling pathways (Low 2005).

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 15

Involvement of Astroglial Fibroblast Growth Factor-2 and Microglia in the Nigral 6-OHDA Parkinsonism and a Possible Role of Glucocorticoid Hormone on the Glial Mediated Local Trophism and Wound Repair

Camila Silva, Kjell Fuxe, and Gerson Chadi

Abstract We have observed in previous studies that 6-hydroxydopamine (6-OHDA)-induced lesions in the nigrostriatal dopamine (DA) system promote increases of the astroglial basic fibroblast growth factor (FGF-2, bFGF) synthesis in the ascending DA pathways, event that could be modified by adrenosteroid hormones. Here, we first evaluated the changes of microglial reactivity in relation to the FGF-2-mediated trophic responses in the lesioned nigrostriatal DA system. 6-OHDA was injected into the left side of the rat substantia nigra. The OX42 immunohistochemistry combined with stereology showed the time course of the microglial activation. The OX42 immunoreactivity (IR) was already increased in the pars compacta of the substantia nigra (SNc) and ventral tegmental area (VTA) 2 h after the 6-OHDA injection, peaked on day 7, and remained increased on the 14th day time-interval. In the neostriatum, OX42 immunoreactive (ir) microglial profiles increased at 24 h, peaked at 72 h, was still increased at 7 days but not 14 days after the 6-OHDA injection. Two-colour immunofluorescence analysis of the tyrosine hydroxylase (TH) and OX42 IRs revealed the presence of small patches of TH IR within the activated microglia. A decreased FGF-2 IR was seen in the cytoplasm of DA neurons of the SNc and VTA as soon as 2 h after 6-OHDA injection. The majority of the DA FGF-2 ir cells of these regions had disappeared 72 h after neurotoxin. The astroglial FGF-2 IR increased in the SNc and VTA, which peaked on day 7. Two-colour immunofluorescence and immunoperoxidase analyses of the FGF-2 and OX42 IRs revealed no FGF-2 IR within the reactive or resting microglia. Second, we have evaluated in a series of

biochemical experiments whether adrenocortical manipulation can interfere with the nigral lesion and the state of local astroglial reaction, looking at the TH and GFAP levels respectively. Rats were adrenalectomized (ADX) and received a nigral 6-OHDA stereotaxical injection 2 days later and sacrificed up to 3 weeks after the DA lesion. Western blot analysis showed time-dependent decrease and elevation of TH and GFAP levels, respectively, in the lesioned versus contralateral midbrain sides, events potentiated by ADX and worsened by corticosterone replacement. ADX decreased the levels of FGF-2 protein (23 kDa isoform) in the lesioned side of the ventral midbrain compared contralaterally. The results indicate that reactive astroglia, but not reactive microglia, showed an increased FGF-2 IR in the process of DA cell degeneration induced by 6-OHDA. However, interactions between these glial cells may be relevant to the mechanisms which trigger the increased astroglial FGF-2 synthesis and thus may be related to the trophic state of DA neurons and the repair processes following DA lesion. The findings also gave further evidence that adrenocortical hormones may regulate astroglial-mediated trophic mechanisms and wound repair events in the lesioned DA system that may be relevant to the progression of Parkinson's disease.

Keywords Parkinson • Astrocyte • Microglia • Dopamine • Fibroblast growth factor-2 • Wound repair • Neuroregeneration

Abbreviations

FGF-2	bFGF, basic fibroblast growth factor
DA	Dopamine
DAB	3, 3' diaminobenzidine tetrahydrochloride
GFAP	Glial fibrillary acidic protein
IR	Immunoreactivity
ir	Immunoreactive
6-OHDA	6-hydroxydopamine
PBS	Phosphate-buffered saline

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SNC	Pars compacta of the substantia nigra
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area.

Introduction

After a lesion applied to the central nervous system, the quiescent microglial and astroglial cells become reactive and start to orchestrate events related to neurodegeneration/neuroprotection as well as wound repair. The reactive microglia, named amoeboid microglia, loses its processes and shows an increased size (Giulian and Baker 1985). Furthermore, the activated microglia starts to phagocytose the lesioned cells as well as to synthesize and to release factors (Giulian and Vaca 1993). It is postulated that the degree of the degeneration of the injured neurons is related to the intensity of the microglial reaction (Giulian 1993; Giulian and Robertson 1990; Giulian and Vaca 1993).

Astroglial activation is also observed following a lesion of the central nervous system (Strömberg et al. 1986). The reactive astrocytes increase in size and synthesize glial fibrillary acid protein (GFAP) and several neurotrophic factors (Baumann et al. 1993; Chadi et al. 1994; Giulian et al. 1993; Martin 1992).

The neuronal survival following an injury can be mediated by paracrine actions of the neurotrophic factors released by the reactive astrocytes (Chadi et al. 1993a; Martin 1992; McMillian et al. 1994; Park and Mytilineou 1992). Several studies have described that the actions of the basic fibroblast growth factor (FGF-2, bFGF) in the central nervous system are mainly mediated by activated astrocytes (Chadi et al. 1994; Finklestein et al. 1988; Frautschy et al. 1991; Humpel et al. 1993, 1994; Pasinetti et al. 1999).

FGF-2 mRNA and protein were demonstrated in neurons and astrocytes of many brain regions (Matsuyama et al. 1992; Woodward et al. 1992). In a previous study, we have observed that the 6-hydroxydopamine (6-OHDA)-induced lesions in the nigrostriatal dopamine (DA) system promote increases of FGF-2 synthesis in the reactive astrocytes in a time-dependent manner in the ascending DA pathways (Chadi et al. 1994). We have also observed that exogenous FGF-2 may protect DA neurons from DA neurotoxicity, in part via the upregulation of astroglial FGF-2 (Chadi et al. 1993a, Chadi and Fuxe 1998).

Opposing actions of the astroglia and microglia have been described in the regulation of the trophic state in the central nervous system (Giulian et al. 1993). Furthermore, modulatory chemical interactions between these glial cells may define whether the central neurons die or survive following an injury and also to the progression of neurodegenerative

diseases such as Parkinson's disease (Giulian et al. 1993; McMillian et al. 1994).

Steroid hormones may regulate the state of glial activation and the expression of glial substances in the lesioned brain, thus playing a role on brain plasticity/trophism as well as wound repair events after injury (Barbany and Persson 1993; Chadi et al. 2008; Chao et al. 1998; Chao and McEwen 1994; Ferrari et al. 2008; Laping et al. 1991; Pollock et al. 1990; Riva et al. 1995a; Smith et al. 1995). Thus, the analysis of the hormonal influences on glial-mediated neurodegenerative/neuroprotection events in the basal ganglia becomes important, with particular interest to pathophysiology of Parkinson's disease.

The present study was performed to evaluate the morphological features of microglial activation in relation to the FGF-2 immunoreactive (ir) cells in the lesioned nigrostriatal DA system, using the single- and two-colour immunofluorescence or immunoperoxidase techniques. The study also evaluated whether a bilateral removal of the adrenal gland can alter the levels of tyrosine hydroxylase (TH), of the glial fibrillary acid protein (GFAP), the major component of the intermediate filament of astroglial cytoskeleton, and the astroglial FGF-2 isoform in nigrostriatal regions after a 6-OHDA-induced degeneration of dopamine neurons.

Methods and Materials

Subjects

Specific pathogen-free adult male Wistar rats (University of São Paulo Scholl of Medicine) weighing 200–250 g were used in this study. The rats were kept under controlled temperature and humidity conditions with standardized lighting time (lights on at 6:00 h and off at 18:00 h) and free access to food pellets and tap water. The use of animals was approved by the regional ethics committee for animal research and followed the guidelines of the national agency for research on animals.

Experiment of Glial Reaction After Nigrostriatal Lesion

Fifty-four rats were anaesthetized with halothane (1.5–3%, Sigma, U.S.A.), placed in a stereotaxic apparatus (David Kopf, U.S.A.) and 6-OHDA hydrochloride (8 µg/µl, Sigma, U.S.A.), dissolved in 0.9% saline containing ascorbic acid (0.2 mg ml⁻¹), was unilaterally injected over a 4 min-interval into the left substantia nigra (*n*=30). The coordinates were

bregma -4.4 mm, lateral 1.2 mm, and ventral 7.8 mm as in a previous description (Chadi et al. 2008). Control rats ($n=24$) received the solvent of the 6-OHDA. Two hours, 4 h, 24 h, 72 h, 1 week, and 2 weeks after the 6-OHDA injection, the animals were anaesthetized with sodium pentobarbital (Mebumal, $100 \text{ mg} \times \text{Kg}^{-1}$, b.wt., i.p.) and processed for immunohistochemistry as described in the following paragraph.

The animals were perfused through a cannula inserted in the ascending aorta with 50 ml isotonic saline followed by 150 ml fixation fluid (4°C) for 6 min. The fixative consisted of 4% paraformaldehyde (w/v) and 0.2% picric acid (v/v) in 0.1 M phosphate buffer (pH 6.9). The brains were removed, kept in the fixative at 4°C for 90 min, and rinsed for 48 h in 10% sucrose dissolved in phosphate-buffer. Coronal frozen sections ($14\mu\text{m}$ thick) were taken from rostro-caudal levels of the midbrain (-5.20 mm to -6.04 mm) and forebrain (-0.30 mm to 1.30 mm), (Paxinos and Watson 1986), using a CM3000 Leica cryostat (Germany). The sections were stored at -70°C until use.

The tyrosine hydroxylase (TH) IR, by means of the single immunofluorescence technique, was employed to monitor the disappearance of the DA cell bodies and terminals. Thaw-mounted sections were incubated overnight at 4°C in a humidified chamber with a rabbit polyclonal TH antibody (Sigma, U.S.A.), diluted 1:400. The antibody was diluted in PBS containing 0.3% Triton X-100 (Sigma, U.S.A.) and 0.5% bovine serum albumine (Sigma). Sections were washed in phosphate-buffered saline (PBS) (3×10 min) and incubated for 45 min at 37°C with a fluoresceine isothiocyanate-conjugated donkey antirabbit antibody (diluted 1:30, Jackson, West Grove, U.S.A.). The sections were rinsed in PBS and mounted in an antifading medium and examined in a Nikon Microphot-FX epifluorescence microscope. Control incubations were made without the primary antibody.

Single immunoperoxidase technique was used to detect the OX42 immunoreactivity (IR), a marker for microglia, by means of the avidin-biotin peroxidase method. Thaw-mounted sections were incubated overnight at 4°C in a humidified chamber with a mouse monoclonal OX42 antibody (diluted 1:2,000, Harlan, U.K.), diluted in the same solvent, as already described. Sections were washed in PBS (3×10 min) and incubated with a biotinylated horse antimouse antibody (diluted 1:200, Vector, U.S.A.) for 1 h. Subsequently, sections were washed in PBS and incubated in the avidin-biotin peroxidase complex (both diluted 1:100, Vectastain, Vector) for 45 min. Visualization of the immunoreactivity was carried out in 0.03% 3, 3' diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen and H_2O_2 (0.05%, Sigma) for 5 min. Control incubations were also performed without the primary antibody. For the standardization of the immunohistochemical procedure, we used a dilution of the primary antibody and a concentration of the

DAB far from saturation and an adjusted incubation time, so that the darkest elements in the brain sections were below saturation.

Two-colour immunofluorescence technique was used for the simultaneous detection of the OX42 and TH IRs as well as the OX42 and FGF-2 IRs in the lesioned areas. (a) *TH and OX42 IRs*. Sections were incubated overnight at 4°C in a humidified chamber with a mixture of the rabbit polyclonal TH (1:400) and the mouse monoclonal OX42 (1:300) antibodies. After several rinses in PBS, the sections were incubated for 45 min at 37°C with a mixture of fluoresceine isothiocyanate-conjugated donkey antirabbit and texas red conjugated antimouse immunoglobulins (both diluted 1:30, Jackson) to demonstrate the TH and OX42 IRs respectively. (b) *FGF-2 and OX42 IRs*. Sections were incubated with a rabbit polyclonal FGF-2 (1:400) and the mouse monoclonal OX42 (1:400) antibodies. After several rinses in PBS, the sections were incubated for 45 min at 37°C with a mixture of fluoresceine isothiocyanate-conjugated donkey antirabbit and texas red conjugated donkey antimouse immunoglobulins (both diluted 1:30, Jackson) to demonstrate the FGF-2 and OX42 IRs, respectively. The sections were rinsed in PBS, mounted in the antifading medium and examined in the epifluorescence microscope. The FGF-2 antiserum is a well-characterized polyclonal antibody raised against the N terminal (residues 1–24) of the synthetic peptide of bovine FGF-2 (1–146) purchased from Sigma (U.S.A.). This antiserum does not recognize acidic FGF (cross reactivity less than 1%) (Chadi et al. 1994).

The two-colour immunoperoxidase method (Gomide and Chadi 1999) was employed in a series of sections for the simultaneous detection of the FGF-2 and the OX42 IRs. This technique was used in the present analysis, since it is more sensitive than the two-colour immunofluorescence technique (Vectastain, Vector, U.S.A.) and because we were not able to stain quiescent microglia using OX42 immunofluorescence. The FGF-2 IR was first demonstrated. Briefly, sections were incubated overnight at 4°C in a humidified chamber with the rabbit polyclonal FGF-2 antiserum, diluted 1:1,000 in the same solvent previously mentioned (Chadi et al. 1993b). After rinsing in PBS, the sections were incubated with biotinylated goat antirabbit immunoglobulins (1:200, Vector, for 1 h), again rinsed in PBS and incubated with the avidin-biotin complex (1:100, Vectastain) for 45 min. The detection of the antibody was done as previously described, using DAB as chromogen. Following the DAB reaction, the sections were rinsed several times in PBS and incubated for 48 h at 4°C in a humidified chamber with the mouse monoclonal OX42 antiserum described previously (diluted 1:400). After several rinses in PBS, the sections were incubated with biotinylated horse antimouse immunoglobulins (diluted 1:200, Vector) for 1 h at room temperature, again rinsed in PBS and incubated with the avidin-biotin

solution (both diluted 1:100, Vectastain, Vector) for 45 min at room temperature. The staining was performed using 0.05% of 4-chloro naphthol (Sigma) as a chromogen and 0.05% (v/v) of H₂O₂ (Sigma) for 10 min. The sections were rinsed in PBS, and coverslipped in a glycerol phosphate buffered medium. This procedure gave a brownish colour to FGF-2 and a bluish colour to OX42 IRs. The sections were analyzed and photographed in a Zeiss photomicroscope (Germany).

As controls, sections were incubated with the FGF-2 anti-serum (diluted 1:700) preincubated with human recombinant FGF-2 (50 µg/ml) for 24 h at 4°C. To further analyze the specificity of the immunostainings, sections were also incubated with the solvent of the primary and secondary antibody solutions as well as the solvent of the avidin–biotin solution and processed simultaneously with the experimental sections.

The Areal Fraction (AA) of the OX42 IR was calculated employing stereological methods in the studied regions (Cerutti and Chadi 2000; Gomide and Chadi 1999). The SNc and VTA ipsilateral to the 6-OHDA injection were analyzed in sections at the bregma level -5.30 mm (Paxinos and Watson 1986) and the ipsilateral neostriatum in sections at the bregma level 0.70 mm. The Areal Fraction of the OX42 IR was calculated using the CAST-system (Computer Assisted Stereological Toolbox, Olympus, Denmark), which created a set of regularly spaced points and a counting frame in fields systematically sampled in the studied regions, as an overlay image to the microscope image on the monitor (Chadi et al. 1994; Gundersen et al. 1988). Briefly, an Olympus BX50 microscope (Olympus, Denmark) was interfaced with a computer (IBM 330-P75, U.S.A.) and a colour video camera (JAL 2040, Protec, Japan), both linked to a colour video monitor (G70, IBM). GRID software package, (Interactivision, Silkeborg, Denmark) generated sampling and point-grid frames as an overlay image to the microscopic image on the monitor (Gundersen et al. 1988) as well as to control the motorized X-Y stage (Lang, Huttenberg, Germany). The SNc and VTA were taken together in the measurements. The border of each region (SNc/VTA and neostriatum) was outlined using a $4\times$ objective and the delineated area was obtained. Step rates were entered ($200\mu\text{m}$ for the SNc/VTA and $400\mu\text{m}$ for the neostriatum), after which the system generated a randomized and systematic sampling within the outlined region. For counting the profiles a $60\times$ objective was used. The points hitting all immunoreactive profiles found in the counting frames were counted ($\Sigma P_{\text{structure}}$). The points hitting the sampled tissue were also counted (ΣP_{tissue}). Thus the Areal Fraction $AA_{(\text{structure/section})}$ could be calculated $AA = \Sigma P_{\text{structure}} / \Sigma P_{\text{tissue}}$. The coefficient of error (CE) for the Areal Fraction measurements of each animal was obtained (Gundersen et al. 1988): $CE = SEM/\text{mean}$.

Biochemical Analysis After Adrenocortical Manipulation

In this set of experiments, rats were divided randomly in three groups: (1) 6-OHDA/SHAM/Veh: sham operated rats for ADX ($n=18$) received nigral 6-OHDA 48 h after surgery (sham operation for adrenalectomy) and daily systemic injections of the vehicle solution of corticosterone; (2) 6-OHDA/ADX/Veh: bilateral adrenalectomized rats ($n=18$) received nigral 6-OHDA 48 h after surgery and daily systemic injections of the vehicle solution of corticosterone; and (3) 6-OHDA/ADX/Cort: bilateral adrenalectomized rats ($n=18$) received nigral 6-OHDA 48 h after surgery and daily systemic injections of corticosterone. The systemic injections and corticosterone replacement are described in the following paragraphs.

Thirty-six rats were submitted to a bilateral ablation of the adrenal gland in the morning and 18 animals were sham operated for adrenalectomy (ADX). Briefly, the rats were anaesthetized with halothane (1.5–3%), the skin of the right side of their flank was opened, the muscles dissected, and the adrenal gland removed. The same procedure was made on the left side. After ADX, the rats received 0.9% saline in the drinking water. The adrenal glands were fixed and examined histologically to ensure their surgical removal. We have demonstrated in a previous work that there are no corticosterone levels in the blood after bilateral ADX (Cintra et al. 1994b). Additionally, 18 rats (SHAM) were submitted to sham operation for ADX.

Sham-operated rats received daily injections of vehicle (6-OHDA/SHAM/Veh, $n=18$) beginning on the same day of the surgery. Moreover, ADX rats were divided in two groups of animals that received daily injections of either vehicle (6-OHDA/ADX/Veh, $n=18$) or $10\text{mg} \times \text{Kg}^{-1}$ b.w., ip. of corticosterone (Sigma, U.S.A) (6-OHDA/ADX/Cort., $n=18$). Corticosterone was suspended in a bidistilled water solution containing carboxymethylcellulose sodium salt (0.25% w/v; Sigma) and polyoxyethylene sorbitol mono-oleate (tween 80, 0.2% v/v; Sigma). Animals received the injections for 2 days before the nigral 6-OHDA and for additional 72 h, 1 week, and 3 weeks after the neurotoxin. All injections were made in the afternoon to mimic the endogenous peak secretions of corticosterone and the solvent was given in the same volume and at the same time as the corticosterone injections. This high dose of corticosterone was chosen, since it is a standard dose used to mimic the stress level of corticosterone (Chadi et al. 1993b).

Twenty-two hours, 1 week, and 3 weeks after the nigral 6-OHDA and after the last systemic injection, the rats of the 6-OHDA/SHAM/Veh, 6-OHDA/ADX/Veh and 6-OHDA/ADX/Cort groups were killed by decapitation. The brains of animals were rapidly removed and the midbrain was

isolated by means of cranial and caudal coronal transections. The region including the substantia nigra and ventral tegmental area was dissected out carefully from the ventral midbrain under a stereomicroscope bilaterally. The tissues were frozen and stored in -70°C freezer until use.

TH, GFAP, and FGF-2 protein levels were assessed by quantitative Western blot analysis. FGF-2 experiments were performed only on the 1 week lesioned rats. Both sides of the ventral midbrain as well as the neostriatum (ipsi and contralateral to the lesion) were individually homogenized in lysis buffer containing 1% protease inhibitor cocktail (Sigma), 1% NP40 (Sigma), 0.5% sodium deoxycholate (Sigma), 1mM EDTA (Sigma), and 1mM EGTA (Sigma), diluted in phosphate buffered saline (PBS, pH 7.4) and then sonicated. The homogenate was centrifuged (14,000rpm) for 20 min at 4°C ; supernatants were transferred into new tubes and stored at -70°C until use.

Protein concentrations were determined (Chadi et al. 2008). The samples (60 μg of protein) were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide (Bio-Rad) by electrophoresis gel. Proteins were transferred onto nitrocellulose membranes (Bio-Rad) for 1 h to 100 V. One hour after blocking with 5% milk in Tris-buffered saline-tween (TBS-T), the membranes were incubated with rabbit antibodies to TH (1:7,500; Sigma), GFAP (1:5,000), α -tubulin (1:10,000, Sigma), or FGF-2 (1:1,000 in 3% milk/TBS-T, Sigma) overnight. The FGF-2 antiserum is a well-characterized antibody raised against the n-terminal synthetic bovine FGF-2 (residues 1–24) (de Levy et al., 2007). The antibodies were described earlier. Membranes were washed 2 times for 10 min in TBS-T and incubated at room temperature for 1 h with a 1:10,000 dilution of antirabbit IgG–ECL conjugated secondary antibody (Santa Cruz Biotechnology, USA). Blots were washed two times with TBS-T and once TBS. After final washes, the membranes were incubated with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science, USA) for 1 min. The membranes were exposed to an X-ray film for imaging (HyperfilmTM ECL, Amersham Biosciences, USA) to visualize protein bands. Protein levels were quantified by densitometry by means of a computer-assisted image analyzer and software developed by Imaging Research (Brock University, Canada).

Statistical Analysis

In the experiments of cellular analysis, statistical evaluation was performed according to the nonparametric two-tailed Mann-Whitney *U*-test. The saline and 6-OHDA -treated groups were compared. In the biochemical experiments of TH and GFAP protein levels, the two-way analysis of variance was applied to compare the ipsilateral and contralateral

sides and the periods after surgery, using the protected Bonferroni test for statistical significances between groups. A p value < 0.05 was used to indicate significant differences between groups. Moreover, the Western blot analysis of FGF-2 levels, the one-way analysis of variance (ANOVA), was applied to compare the groups at the ipsilateral lesioned side and at the contralateral side of nigral injection as well as ipsilateral side between the groups, using the protected Tukey test for statistical significances between groups.

Results

Cellular Analysis in the Lesioned Nigrostriatal Pathway

The TH IR was decreased in the neuropil of the ipsilateral SNc and VTA 4 h after the unilateral 6-OHDA injection on the left side of the substantia nigra. There was a marked disappearance of the TH immunoreactive cell bodies in the ipsilateral SNc and VTA, 72 h after the injection, which was complete by 1 week (Fig. 1a). One or two weeks after the 6-OHDA injection, only scattered TH ir cell bodies were found in the ipsilateral pars compacta of the substantia nigra and VTA (Fig. 1f, h, j, l). In the ipsilateral neostriatum, the density of TH ir nerve terminals decreased at 4 h and they had disappeared 1 week after the unilateral nigral injection of 6-OHDA (not shown).

Quiescent OX42 immunoreactive microglial profiles with small cytoplasm and processes were found throughout the studied region of the solvent-injected rats (not shown) as well as in the substantia nigra, VTA, and forebrain regions of the contralateral side of the neurotoxin injection in the analyzed time-interval (Fig. 1b–d). On the lesioned side, the degeneration of the DA pathway was paralleled by a microglial reaction as seen by the increased OX42 IR in the lesioned areas (Figs. 1–4). The OX42 immunoperoxidase revealed increases in the density of the profiles as well as in the size of the cytoplasm and the cytoplasmic processes of the OX42 ir profiles in the SNc and VTA that were already apparent 2 h after the 6-OHDA injection (Fig. 5). In the SNc and VTA, many of OX42 ir profiles had lost their processes and gained a rounded shape 72 h after the 6-OHDA injection and the overall OX42 IR increased until day 7 after the lesion (Figs. 1 and 2). On the lesioned side of the neostriatum, the OX42 ir profiles with processes were observed to be increased in size and density 24 h after the 6-OHDA injection. The neostriatal OX42 ir profiles showed further increases in size and density until 72 h after the lesion (Fig. 3). After this time-point, the microglial reaction declined in the neostriatum. Round OX42 ir profiles in the lesioned neostriatum were not observed at any time-point studied (Fig. 3).

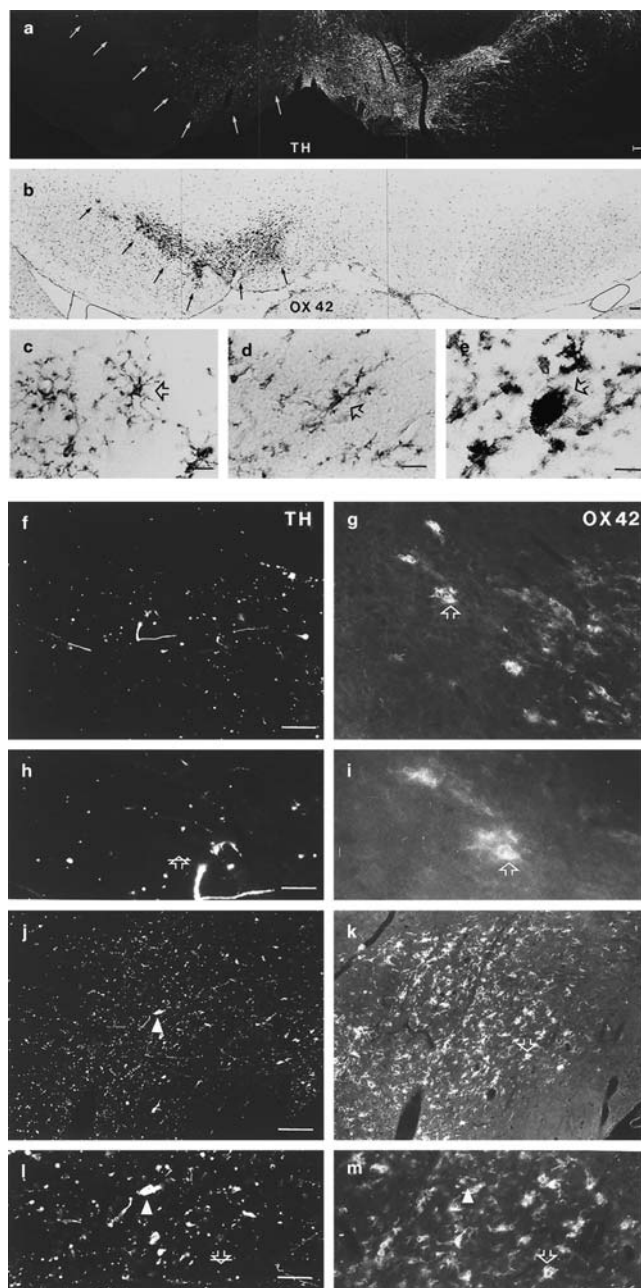


Fig. 1 Indirect immunofluorescence (a) and immunoperoxidase (b–e) procedures have been used for the demonstration of TH (a) and OX42, as a marker for microglia, (b–e) immunoreactivities in the rat brain following a 7 day 6-OHDA stereotaxic injection on the left substantia nigra. An almost complete disappearance of the TH positive cell bodies and dendrites is observed in the substantia nigra and ventral tegmental area ipsilateral to the injection (surrounded by arrows in a) Increased OX42 immunoreactivity is seen in the lesioned pars compacta of the substantia nigra and ventral tegmental area (surrounded by arrows B). Quiescent microglial cells (opened arrows) taken from the unlesioned side of the cerebral cortex (c) and the pars compacta of the substantia nigra (d) are indicated by open arrows. Ameboid reactive microglia taken from the lesioned pars compacta of the substantia nigra is indicated by an open arrow in (e) Scale bars: 100µm (a, b); 50µm (C–E). Bregma=5.80 mm. Two colour immunofluorescence for the simultaneous detection of the tyrosine hydroxylase (TH) (f, h, j, l) and OX42 (g, i, k, m) immunoreactivities (IRs) in the pars compacta of the substantia nigra (f–i) and ventral tegmental area (j–m) of a 1 week 6-OHDA lesioned rat. Figures h, i and l, m represent high magnification of areas from f, g and j, k, respectively. A disappearance of the TH and an increased OX42 (open arrows) IRs are seen in the lesioned pars compacta of substantia nigra and ventral tegmental area. Scattered TH immunoreactive cell bodies resistant to the lesion (arrowheads, j, L) are shown. The crossed open arrows represent the position of the OX42 immunoreactive profiles in the TH immunoreactive panels (h, l) and the crossed arrowhead shows the position of a remaining TH immunoreactive profile in the OX42 immunoreactive panel (m). Scale bars: 100µm (f, j), 50µm (h, l). Bregma=–5.80 mm

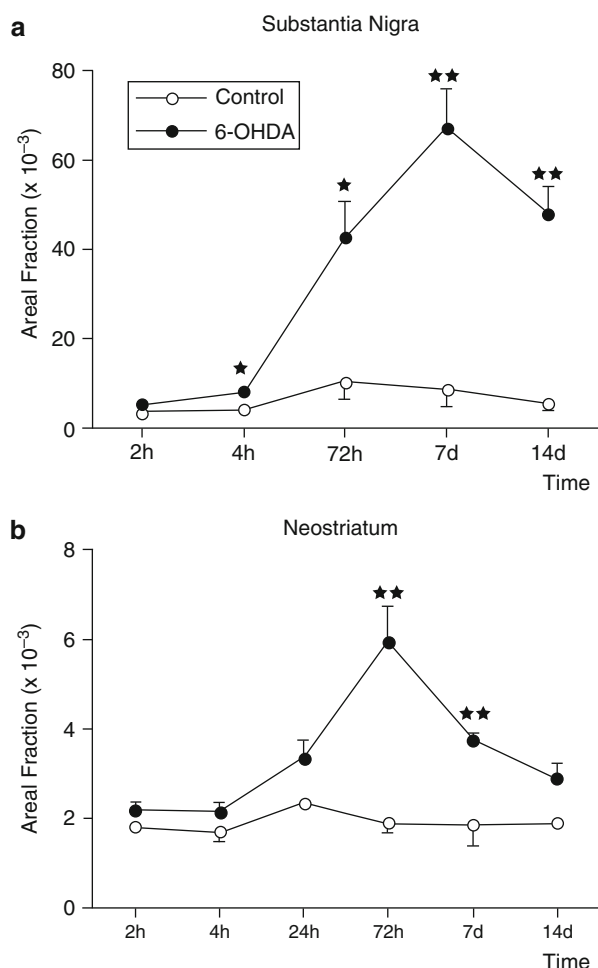


Fig. 2 The stereological method was used to demonstrate the time-course of the microglial activation in the ipsilateral pars compacta of the substantia nigra and ventral tegmental area (a) and in the ipsilateral neostriatum (b) of rats that received a 6-OHDA ($n=5$) or saline (control $n=4$) injection on the left substantia nigra. The numbers represent the mean $\pm$ s.e.m of the Areal Fraction obtained from each animal. * $p < 0.05$, ** $p < 0.01$, according to the Mann-Whitney U -test

The quantitative analysis of the Areal Fraction of the OX42 immunoreaction in the SNc and VTA showed an increased OX42 ir area from 4 h (99.6%) to 1 week (313.5%) ipsilateral to the 6-OHDA injection (Fig. 2). In these regions, the Areal Fraction of the OX42 IR remained elevated until week 2 (765.5%), the last time-point studied (Fig. 2). The Areal Fraction of the OX42 IR started to increase by 24 h (43.3%) in the lesioned neostriatum, peaked at 72 h (218.3%) and declined toward control values 1 week (102.6% increased) after the 6-OHDA injection. The Areal Fraction of the OX42 IR in the lesioned neostriatum was still slightly elevated 2 weeks after the 6-OHDA injection but did not reach significance (Fig. 2). The CE group values (Gomide and Chadi, 1999) related to the stereological procedures employed in the Areal Fraction measurements

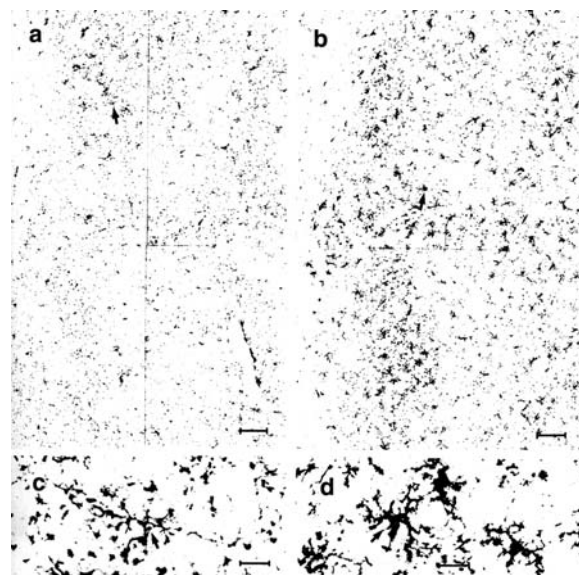
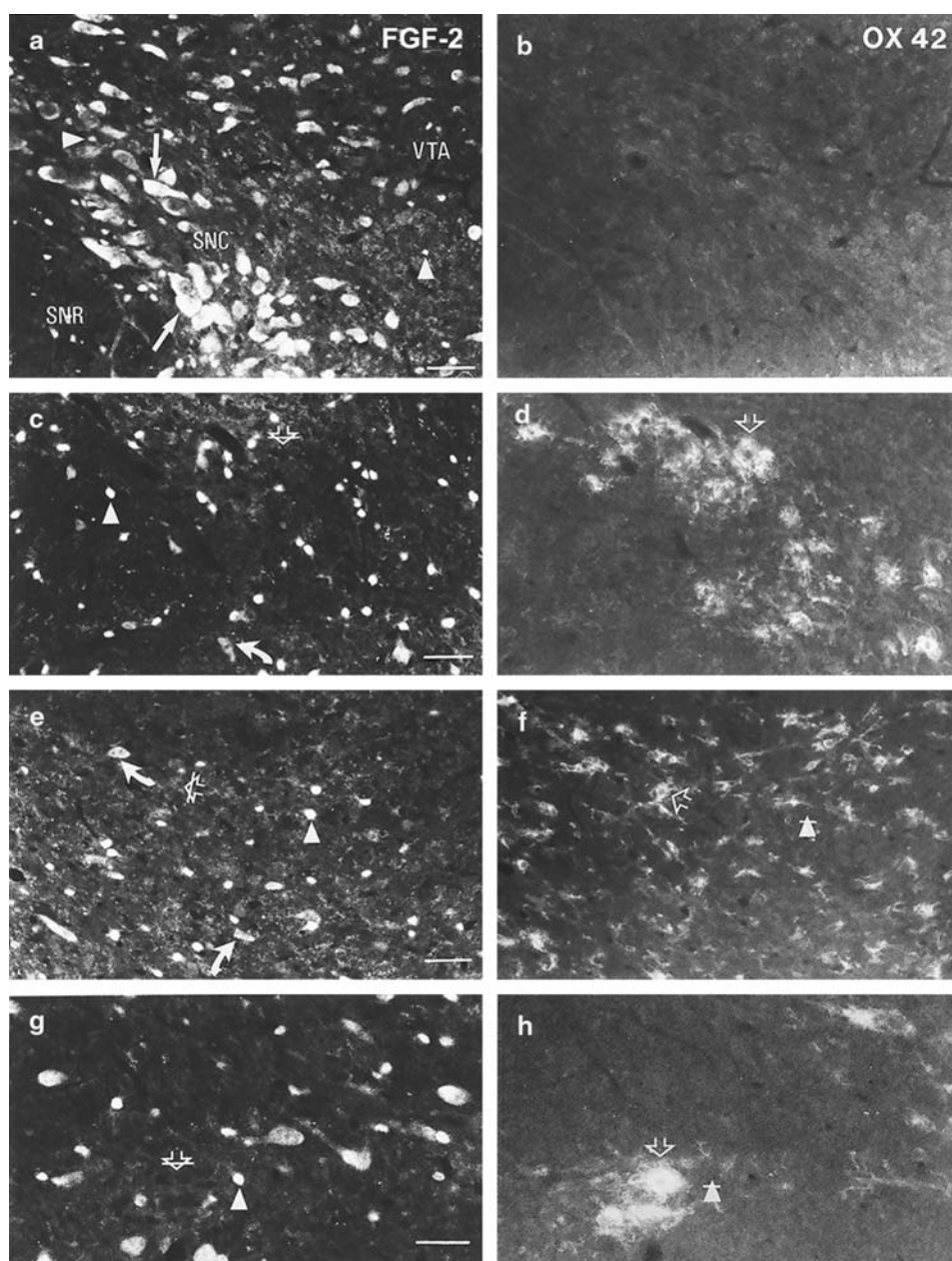


Fig. 3 Photomicrographs showing OX42 immunoreactivity in the ipsilateral neostriatum of the saline (a, c) and 6-hydroxydopamine lesioned (b, d) rat. The injections were made on the left side of the ventral midbrain and the rats were killed 72 h later. OX42 immunoreactivity (arrows) is found in the resting (a–c) and activated (b–d) microglia in the saline and 6-hydroxydopamine injected rats, respectively. Scale bars: 100 μ m (a, b); 20 μ m (c, d). Bregma = -5.80 mm

varied from 0.04 to 0.06 in the studied time-intervals of control and 6-OHDA groups.

The two-colour immunofluorescence for simultaneous demonstration of the OX42 and TH IRs showed an intense microglial reaction close to the DA nerve cell bodies and neuropil of the SNc and VTA (Fig. 6a, b). Degenerated material showing TH IR was found within the OX42 ir profiles (Fig. 6a, b). Moreover, the two-colour immunofluorescence for the simultaneous detection of the FGF-2 and OX42 IRs revealed a decreased FGF-2 IR in the cytoplasm of putative DA neurons of SNc and VTA 2 h after the 6-OHDA injection (not shown). A disappearance of the cytoplasmic FGF-2 ir neuronal profiles was observed in the SNc and VTA 1 week after the 6-OHDA injection (Fig. 4c, e, g). An increased density and intensity of the glial FGF-2 ir nuclear profiles were seen close to the reactive OX42 ir profiles in the SNc and VTA 72 h after the injection. The increased density of the glial FGF-2 ir nuclear profiles peaked at week 1 (Fig. 4) and was still increased 2 weeks after the 6-OHDA injection. No FGF-2 IR was found within the OX42 ir profiles (Fig. 4c–h). Furthermore, OX42 immunofluorescence analysis has not shown quiescent OX42 ir microglia in the SNc, VTA, and neostriatum contralateral to the lesion, or in other unlesioned regions of the central nervous system of the control solvent-injected rats (Fig. 4b). OX42 immunofluorescence was, however, observed in the reactive microglia in the areas close to the needle track of the control rats (not shown).

Fig. 4 Double immunofluorescence procedures have been used to colocalize FGF-2 (bFGF, **a**, **c**, **e**, **g**) and OX42 (**b**, **d**, **f**, **h**) immunoreactivities (IRs) in the pars compacta of the substantia nigra (**a–d**) and the ventral tegmental area (**e–h**) in a coronal section of the adult rat brains submitted to a nigral injection of saline (**a**, **b**) and 6-OHDA (**c–h**). The rats were sacrificed 7 days after 6-OHDA injection. In a saline injected rat (**a**) the FGF-2 IR is observed in the cytoplasm of neurons (arrows) and in the nuclei of glial cells (arrowhead) of the pars compacta of the substantia nigra (SNc) and ventral tegmental area (VTA). OX42 IR is not seen by this method in the saline injected rat (**b**). In a 6-OHDA treated rat only few remaining neurons (curved arrows) show FGF-2 IR. However, an increased FGF-2 IR is observed in the nuclei of glial cells (arrowheads) (**c**, **e**, **g**). An increased OX42 IR is seen in the lesioned areas (open arrows). FGF-2 IR was not colocalized with OX42 IR (cut arrowheads and cut open arrows). Scale bars: 50 μ m (**a–e**); 45 μ m (**g**). Bregma = -5.80 mm

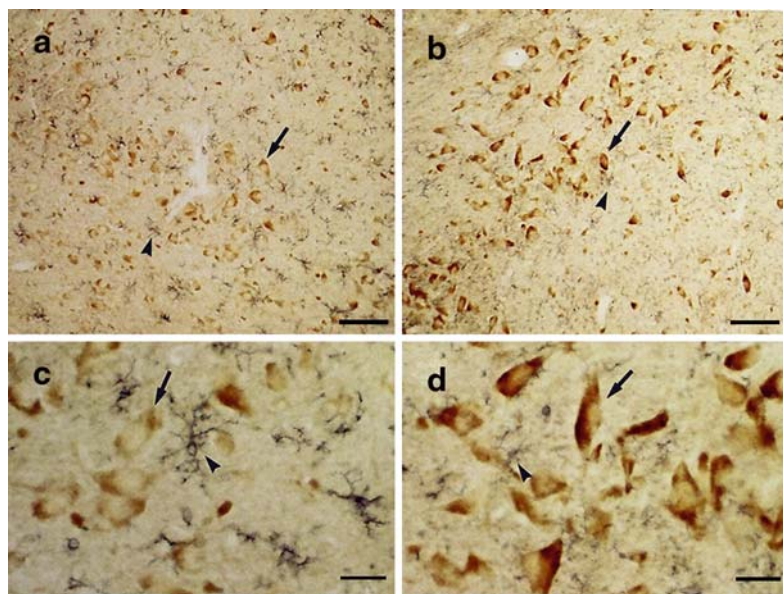


The two-colour immunoperoxidase method for the simultaneous detection of the FGF-2 and OX42 IRs (Figs. 5, 6c–i) was also used, because we could not demonstrate OX42 ir quiescent microglia using the immunofluorescence method. The results confirmed the decreased FGF-2 IR in the cytoplasm of DA neurons of the SNc and VTA 2 h after the 6-OHDA injection (Fig. 5) already observed with immunofluorescence. One week after the 6-OHDA lesion, the majority of the FGF-2 ir profiles disappeared from the SNc and VTA (Fig. 6c, d). We have not found FGF-2 IR within either the quiescent or the reactive OX42 ir profiles in

the studied regions (Fig. 6c–i). However, the presence of activated astrocytes showing increased nuclear FGF-2 IR was frequently observed close to the activated OX42 ir profiles in the SNc and VTA (Fig. 6c–f). In unlesioned regions like cerebral cortex, hippocampus, and the neostriatum of the contralateral side of the unlesioned rats, quiescent OX42 ir profiles close to nuclear FGF-2 ir glial profiles were also commonly seen (Fig. 6g–i).

Sections incubated with the FGF-2 antibody preabsorbed with human recombinant FGF-2 showed no immunoreactivity in neuronal and glial cells. Furthermore, sections

Fig. 5 Photomicrographs showing two-colour immunoperoxidase procedures for simultaneous detection of the FGF-2 (brownish color) and OX42 (bluish colour) IRs in coronal section of the pars compacta of the substantia nigra of the rat brain that have received a nigral injection of 6-OHDA (a, c) or saline (b, d) 2 h before killing. A decreased FGF-2 immunoreactivity (arrows) is seen in the cytoplasm of neurons (a–c) of the 6-OHDA lesioned rats. An increased size of the OX42 immunoreactive profiles (arrowheads) is already observed 2 h after the 6-OHDA injection (a–c). Scale bars: 100 μ m (a, c), 40 μ m (b–d). Bregma=5.80 mm



incubated with the solvent of the primary and secondary antibody solutions or the avidin-biotin solution did not show labeling (data not shown).

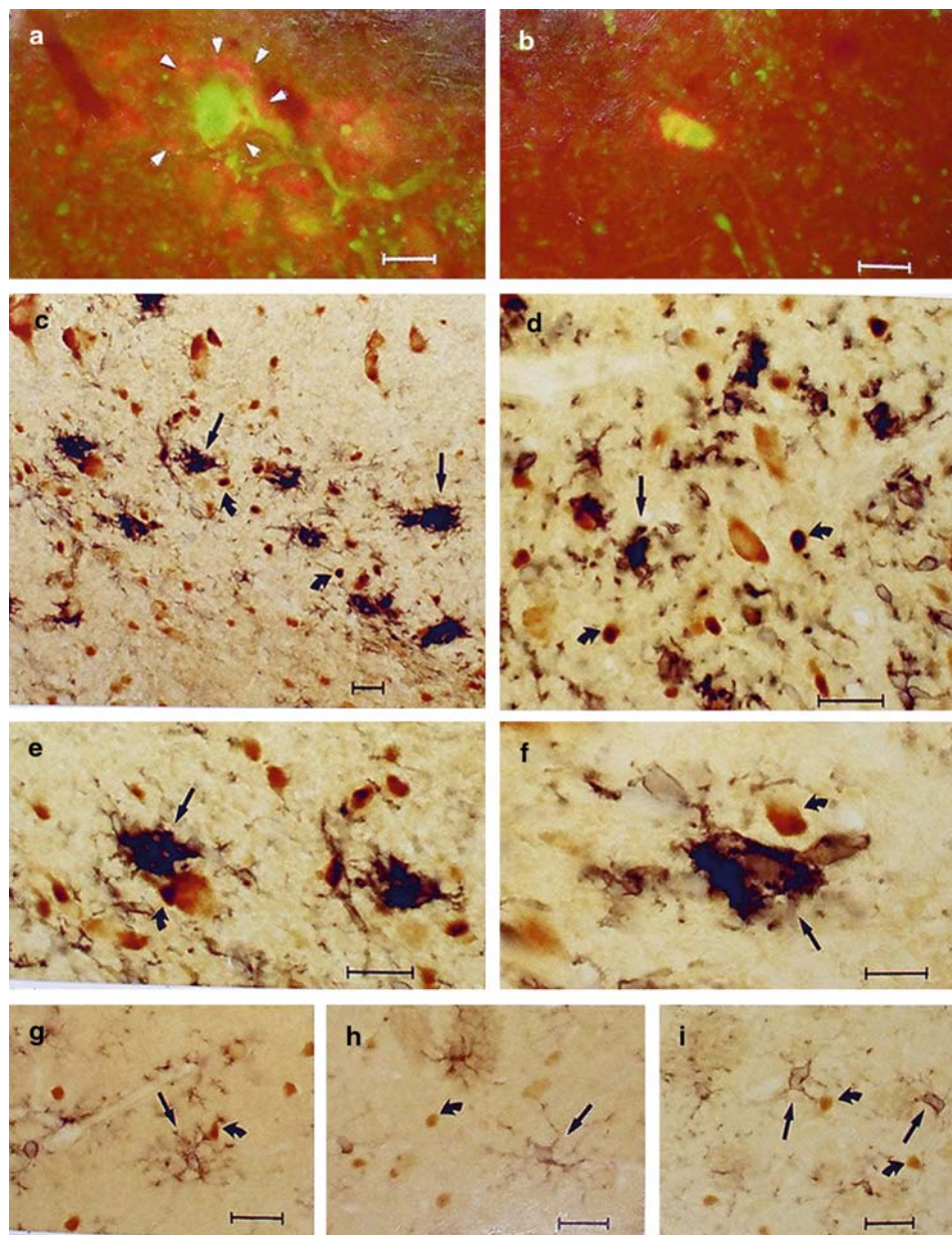
Western Blot Analyses of TH, GFAP and FGF-2 Levels

Injection of 6-OHDA on the left side of the substantia nigra of the rats of the 6-OHDA/SHAM/Veh, 6-OHDA/ADX/Veh, and 6-OHDA/ADX/Cort groups decreased massively the levels of TH in the ipsilateral ventral midbrain, respectively, by 74.69, 82.75, and 87.88% at 72 h time-interval; 90.32, 91.98, and 89.53% at 1 week time-interval; and by 84.97, 84.29, and 94.17% at the third week compared with the same region of the contralateral side (Fig. 7a), and also in the ipsilateral striatum by 61.68%, 72.71%, and 79.95% at 72 h time-interval; 60.89, 70.38, and 62.78% at 1 week time-interval; and by 66.74, 79.93, and 80.66% at the third week compared with the contralateral side (Fig. 7b). Moreover, regarding the TH levels in the ventral midbrain, the two-way ANOVA showed interactions of treatment $\times$ time effects between 6-OHDA/SHAM/Veh and 6-OHDA/ADX/Veh ($p < 0.05$; $F_{3,846}$), between 6-OHDA/SHAM/Veh and 6-OHDA/ADX/Cort ($p < 0.001$; $F_{10,05}$), and between 6-OHDA/ADX/Veh and 6-OHDA/ADX/Cort groups ($p < 0.05$; $F_{4,841}$). However, at the 3 week time-interval, only 6-OHDA/ADX/Cort group differed from 6-OHDA/SHAM/Veh group and from 6-OHDA/ADX/Veh group and also only 6-OHDA/ADX/Veh did not have the level of TH in the ipsilateral/contralateral side, differing in the respective values of the 72 h time-interval (Fig. 7a). The differences at

72 h time-interval are shown in Fig. 7a. Furthermore, regarding the TH levels in the striatum, the two-way ANOVA showed no interactions of treatment $\times$ time effects among groups; however, a trend for difference was found in the temporal effects of treatments on the ipsilateral striatal levels of TH ($p = 0.07$). Fig. 7c illustrates the 60kDa band of the TH by means of Western blot of the ventral midbrain and striatum in the ipsilateral and contralateral sides to the regions at the 72 h time-interval. No changes were detected in the 60kDa α -tubulin bands among groups.

Injection of 6-OHDA on the left side of the substantia nigra of the rats of the 6-OHDA/SHAM/Veh group elevated the levels of GFAP in the ipsilateral ventral midbrain by 6 and 45.11%, respectively, at 72 h and 1 week time-intervals compared with the same region of the contralateral side (Fig. 7a). Ipsilateral increases of 34.09 and 60.37% were found in the 6-OHDA/ADX/Veh and 6-OHDA/ADX/Cort groups, respectively, by 1 week (Fig. 8a). No side differences were found in the striatal levels of the GFAP protein in the ipsilateral side compared with the contralateral side in the studied groups at the analyzed periods (Fig. 8b). Moreover, regarding the GFAP levels in the ventral midbrain and also in the striatum, the two-way ANOVA showed no interactions of treatment $\times$ time effects among groups. However, the statistical analysis also showed differences in the temporal effects of treatments on the ventral midbrain levels of GFAP [between 6-OHDA/SHAM/Veh and 6-OHDA/ADX/Veh ($p < 0.01$; $F_{5,68}$), between 6-OHDA/SHAM/Veh and 6-OHDA/ADX/Cort ($p < 0.01$; $F_{6,324}$), and between 6-OHDA/ADX/Veh and 6-OHDA/ADX/Cort groups ($p < 0.001$; $F_{9,712}$)], but only trends for differences were obtained on the striatal levels of GFAP [between 6-OHDA/SHAM/Veh and 6-OHDA/ADX/Veh ($p = 0.07$), between 6-OHDA/

Fig. 6 Rats received a ipsilateral nigral injection of 6-OHDA (a–f) or saline (g–i) 1 week before killing. Double-immunofluorescence procedures have been used to colocalize tyrosine hydroxylase (TH) (green) and OX42 (red) immunoreactivities in the pars compacta of the substantia nigra of a 1 week 6-OHDA lesioned rat (a, b). In **A** OX42 immunoreactive (ir) processes (arrowheads) surround the TH ir cell bodies and in **b** the TH immunoreactivity (green) is seen as a phagocytic product within the OX42 immunoreactive profile (red). The two-colour immunoperoxidase technique was used for the detection of the FGF-2 (brownish colour, curved arrows) and OX42 (dark bluish colour, straight arrows) **JR** in the pars compacta of the substantia nigra (c, e) and the ventral tegmental area (d, f) ipsilateral to the 6-OHDA nigral injection. The two-colour immunoperoxidase technique was also employed to show FGF-2 (curved arrows) and OX42 (straight arrows) immunoreactivity in the cerebral cortex (g), the hippocampus (h) and the neostriatum (i) of the contralateral side of rats. Bars: 25 μ m (a–e and g–h); 12,5 μ m (f)



SHAM/Veh and 6-OHDA/ADX/Cort ($p=0.05$), and between 6-OHDA/ADX/Veh and 6-OHDA/ADX/Cort groups ($p=0.05$]. The differences of the values compared with correspondent values at the 72 h time-interval are shown in Fig. 8 ($\dagger p<0.05$). Fig. 8c illustrates the 53kDA band of the GFAP by means of Western blot of the Ventral Midbrain and Striatum at the 1 week time-interval in the ipsilateral and contralateral sides to the regions.

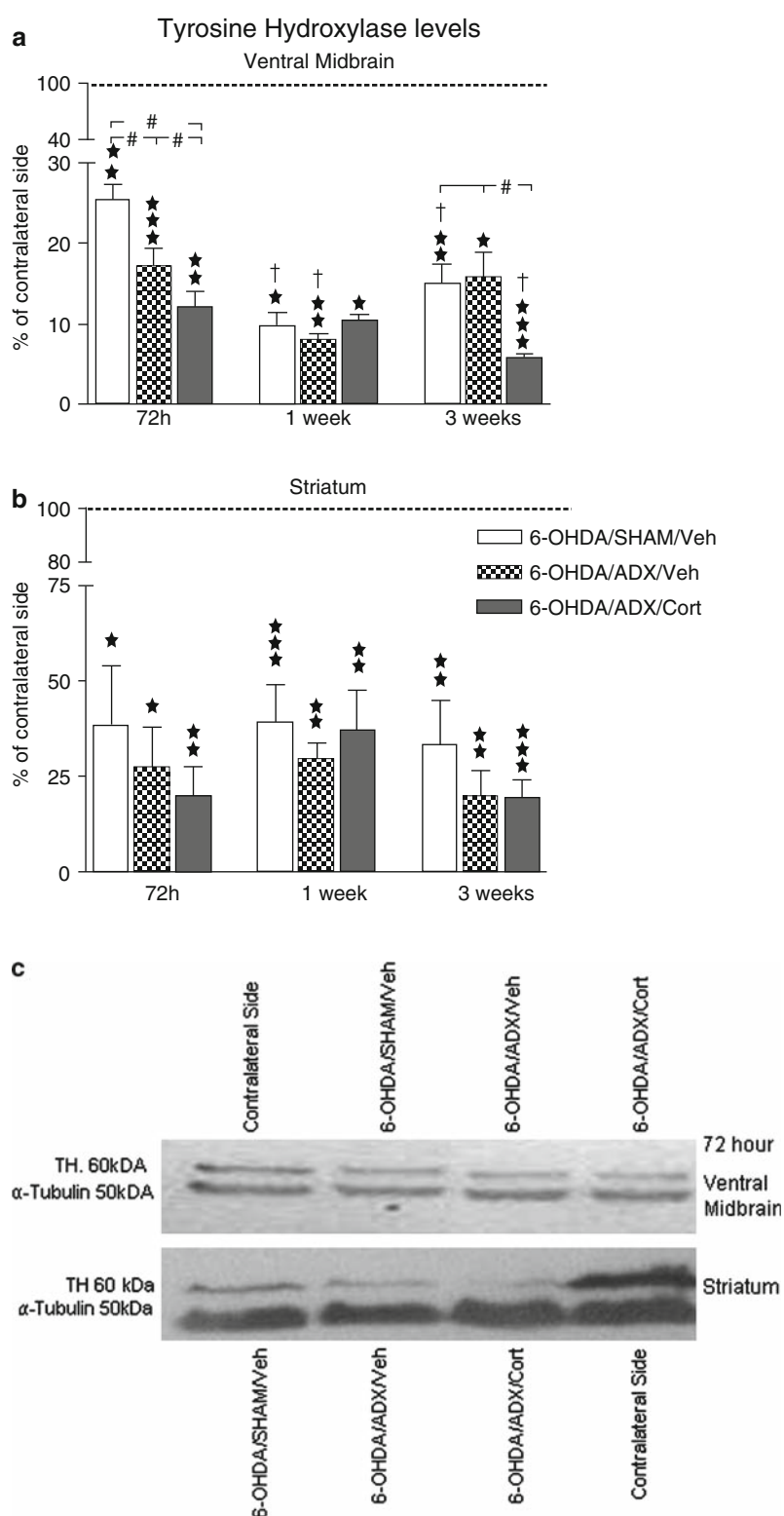
The ADX reduced the levels of the 23kDA isoform of the FGF-2 in the ipsilateral ventral midbrain compared with the contralateral side of the 6-OHDA/ADX/Veh rats ($p<0.05$), an effect that was not reversed by the corticosterone replacement in the 6-OHDA/ADX/Cort animals (Fig. 9a). Moreover, only the 6-OHDA/SHAM/Veh group had the value

of the ipsilateral side different from the contralateral side ($p<0.05$, Fig. 9a). No difference was found in the 21kDA isoform (Fig. 9b).

Discussion

Interactions of reactive glial cells, mainly the astrocytes and microglia, may interfere with the process of DA degeneration, thus playing an important role in the evolution of neurodegenerative diseases such as Parkinson's diseases. This paper has studied the microglial reaction following the 6-OHDA induced nigral lesion in the ascending meso-striatal

Fig. 7 The figure shows the tyrosine hydroxylase (TH) protein levels (relative optical density in **a**, **b** and illustrated in **c**) by means of Western blot. The values represent the percent (Mean $\pm$ s.e.m.) of the ipsilateral rat Ventral Midbrain (**a**) and Striatum (**b**) in relation to the contralateral unlesioned side following an unilateral nigral injection of 6-hydroxydopamine (6-OHDA). Nigral 6-OHDA injected rats were sham operated for bilateral adrenalectomy (ADX) and received daily injection of vehicle (6-OHDA/SHAM/Veh, $n=18$). Moreover, an additional number of 6-OHDA injected rats received ADX and were treated with daily injection of vehicle (6-OHDA/ADX/Veh, $n=18$) or corticosterone (6-OHDA/ADX/Cort, $n=18$). Rats were sacrificed 72 h, 1 week and 3 weeks after nigral neurotoxin. Two-way analysis of variance (ANOVA) was applied for comparisons ipsilateral and contralateral sides ($*p<0.05$, $**p<0.01$, $***p<0.001$) and the periods after surgery (differences described in the text), using the protected Bonferroni test for statistical significances between groups. Differences among groups in each time-interval are pointed ($\#p<0.05$). The differences between the 1 week time-interval or 3 weeks time-interval with the 72 h time-interval are pointed in the figure ($\dagger p<0.05$)



DA pathways of the rat by means of OX42 immunohistochemistry. The relation of the microglial reaction to the increases of astroglial FGF-2 IR in the lesioned DA system was also analyzed.

The microglial reaction was most pronounced close to the degenerating DA cell bodies. We have observed in the SNc and VTA, an activation of OX42 in microglia as soon as 2 h after the 6-OHDA injection as seen by the increased

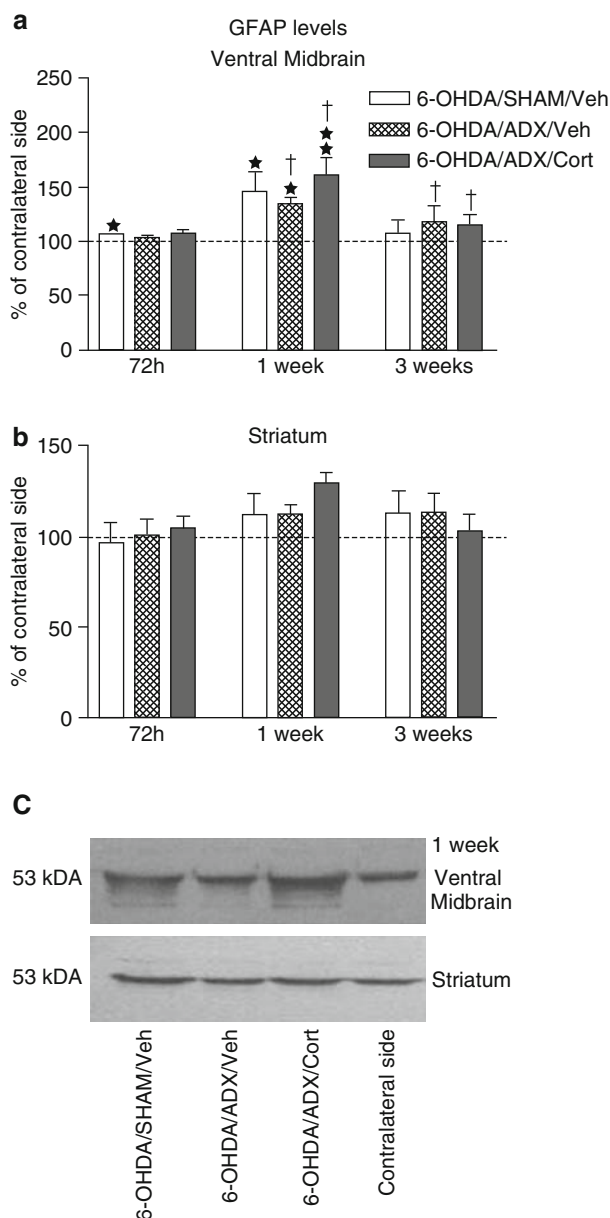


Fig. 8 The figure shows the glial fibrillary acidic protein (GFAP) levels (relative optical density in a, b and illustrated in c) by means of Western blot. The values represent the percent (Mean $\pm$ s.e.m.) of the ipsilateral rat Ventral Midbrain (a) and Striatum (b) in relation to the contralateral unlesioned side following an unilateral nigral injection of 6-hydroxydopamine (6-OHDA). Nigral 6-OHDA injected rats were sham operated for bilateral adrenalectomy (ADX) and received daily injection of vehicle (6-OHDA/SHAM/Veh, $n=18$). Moreover, an additional number of 6-OHDA injected rats received ADX and were treated with daily injection of vehicle (6-OHDA/ADX/Veh, $n=18$) or corticosterone (6-OHDA/ADX/Cort, $n=18$). Rats were sacrificed 72 h, 1 week and 3 weeks after nigral neurotoxin. Two-way analysis of variance (ANOVA) was applied for comparisons ipsilateral and contralateral sides ($*p<0.05$, $**p<0.01$) and the periods after surgery (differences described in the text), using the protected Bonferroni test for statistical significances between groups. The differences between the 1 week time-interval or 3 weeks time-interval with the 72 h time-interval are pointed in the figure ($\dagger p<0.05$)

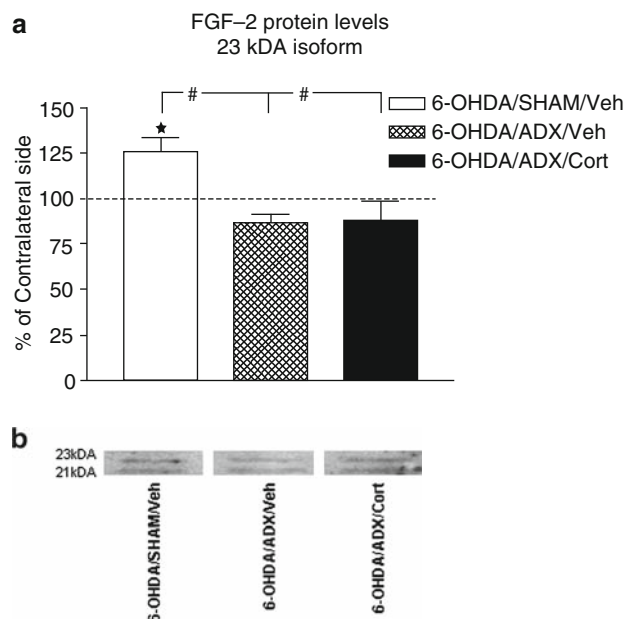


Fig. 9 The figure shows the 23 kDa isoform of the fibroblast growth factor-2 (FGF-2) protein levels (A and illustrated in B) levels by means of Western blot. The values represent the percent (Mean $\pm$ s.e.m.) of the ipsilateral rat Ventral Midbrain in relation to the contralateral unlesioned side following an unilateral nigral injection of 6-hydroxydopamine (6-OHDA). Nigral 6-OHDA injected rats were sham operated for bilateral adrenalectomy (ADX) and received daily injection of vehicle (6-OHDA/SHAM/Veh, $n=6$). Moreover, an additional number of 6-OHDA injected rats received ADX and were treated with daily injection of vehicle (6-OHDA/ADX/Veh, $n=6$) or corticosterone (6-OHDA/ADX/Cort, $n=6$). Rats were sacrificed 72 h after nigral neurotoxin. The one-way analysis of variance (ANOVA) was applied to compare the groups at the ipsilateral lesioned side and at the contralateral side of nigral injection ($*p<0.05$) as well as ipsilateral side between the groups ($\#p<0.05$), using the protected Tukey test for statistical significances between groups. The measurements of the 21 kDa isoform of the protein showed no group differences

size of their cytoplasm and processes. In the SNc and VTA, the reactive OX42 ir profiles gained a round shape and lost their thin processes 72 h after the lesion. In the neostriatum, there was a massive degeneration of the densely packed DA nerve terminals. However, the subsequent microglial reaction in the neostriatum was less intense. In the DA regions of the ventral midbrain, the reaction was also long-lasting, contrasting to the short-lasting microglial activation observed in the neostriatum, associated with the anterograde degeneration of the DA terminals. The microglial activation following the 6-OHDA injection, as seen by the increased size and altered shape of the OX42 ir profiles, was also faster in the SNc and VTA than the neostriatum, probably related to a delay in the onset in the anterograde degeneration.

These findings are in full agreement with previous observations that have demonstrated a massive and long-lasting activation of microglia after mechanical (Jensen et al. 1994;

Leong and Ling 1992; Stichel and Müller, 1994), chemical (Mitchell et al. 1993; Niquet et al. 1994) or ischemic (Finsen et al. 1993; McCall et al. 1995; McRae et al. 1995; Soriano et al. 1994) lesions of the central nervous system. Activated microglia were also described in many neurodegenerative diseases (McGeer et al. 1993). Once activated, microglia are transformed into intrinsic brain macrophages that release several secretory products (Banati et al. 1993).

The mononuclear phagocytes are the dominant effector cell during acute brain injury, which include the invading macrophages (Akiyama and McGeer 1989) and activated microglia, depending on the type of injury (Giordana et al. 1994; Giulian 1987). However, microglia and not macrophages are the readily responding cells after the adult lesion (Milligan et al. 1991). The OX42 antibody employed in this analysis recognizes the complement receptor type 3 (CR3), an immunologically important surface molecule of the mononuclear phagocytes. The ramified resting microglia of the neuronal parenchyma possess proliferate capability and are converted to reactive microglia following brain lesion (Glenn et al. 1992). We cannot exclude that part of the reactive OX42 ir round cells in the 6-OHDA lesioned SNc and VTA represent forms of blood/monocytes macrophages that have transiently entered the brain (Akiyama and McGeer 1989; Ogawa et al. 1993). It might be possible that the 6-OHDA induced-nigral lesion also leads to a blood-brain barrier breakdown with subsequent penetration of the extrinsic mononuclear phagocytes.

It is likely that the majority of the round shape OX42 ir profiles found in the SNc and VTA represents the ameboid microglia (Ling and Wong 1993; Ling et al. 1990; Streit et al. 1988), a state of reactive microglia with phagocytic and immunomodulator properties, which secrete factors like transforming growth factor, colony-stimulating factor, and interleukins (Giulian and Ingeman 1988; Hetier et al. 1988; Morgan et al. 1993). This paper demonstrated that the ameboid microglia readily phagocyte the DA cell bodies and TH IR was observed as a phagocytic product in the cytoplasm of the activated OX42 ir cells.

Extensive and long-lasting changes of glial cells were described following the transection of the postcommissural fornix in the adult rat which included a very early microglial activation, as seen by the increased OX42 IR followed by an astrocytic reaction. The microglia proliferate by cell division and astrocytosis is a main result of the migration of astrocytes and/or the upregulation of the expression of GFAP molecule (Matsumoto et al. 1992).

An astroglial reaction was observed in the SNc and VTA of the ventral midbrain and in the neostriatum following the 6-OHDA induced dopaminergic lesion (Chadi et al. 1994; Pasinetti et al. 1999; Sheng et al. 1993; Strömberg et al. 1986). The activation of the astroglia was more pronounced close to degenerating DA cell body compared with the less

intense but also prominent astroglial reaction in the neostriatum. It was observed that the astroglial reaction starts 24 h after the 6-OHDA lesion, increases until the end of the first week, and then declines (Rataboul et al. 1989; Strömberg et al. 1986). However, it has been described that the reaction is still observed for several months after the lesion of the DA pathway (Strömberg et al. 1986).

Activated microglia was observed in the SNc and VTA 2 h after the 6-OHDA lesion, which preceded the astrocytic reaction both in the DA regions of the ventral midbrain and neostriatum (unpublished observations). The delayed astrocytic reaction was described following chemical (Niquet et al. 1994) and traumatic (Stichel and Muller, 1994) lesions of the central nervous system.

It is possible that in the present lesion the injured DA neurons have triggered signals (Banati et al. 1993) that have activated the surrounding microglia. The locally reactive microglia has engulfed the lesioned DA neurons, cleaned the debris, and also induced the activation of astrocytes *via* astroglia-promoting growth factors, including IL-1 (Fagan and Gage, 1990; Giulian and Baker 1985; Hetier et al. 1988; Wu et al. 1998), or of glutamate synthesis (Piani et al. 1992). Furthermore, the reactive astrocytes may also have released molecules that can further stimulate the microglial reaction (Hao et al. 1990; Malipiero et al. 1990).

Giulian and coworkers (1993) have distinguished the influences of astroglia and microglia on neuronal trophism by monitoring the effects of their secretion products on neuronal survival. It has been described that the actions of FGF-2 in the nigrostriatal DA system are mediated through the activation of astrocytes (Chadi et al. 1993a, Gaul and Lübbert 1992; Park and Mytilineou 1992). It is possible that the interaction between activated astroglia and microglia has triggered the increased synthesis of astroglial FGF-2 (Chadi et al. 1994) or other neurotrophic factors following the DA lesion, in an attempt to maintain the DA neurons (McNaught and Jenner 1999). Thus, evidence has been obtained that following a lesion in the central nervous system, the interaction of the reactive astrocyte and microglia may be involved in vivo in repair and the survival of adjacent neurons in a paracrine fashion (Banati et al. 1993; Baumann et al. 1993; Giulian et al. 1993).

In this study using the two-colour immunoperoxidase technique that is more sensitive than the two-colour immunofluorescence, we could not find the presence of the FGF-2 IR within the OX42 ir profiles. The FGF-2 mRNA and protein were demonstrated in microglial cultures derived from both neonatal rat and human fetal brains (Presta et al. 1995; Shimojo et al. 1991). We cannot exclude that immature microglia can synthesize FGF-2. However, in this study, we have not found FGF-2 IR either in activated or in resting microglia in adult rat brain. However, the presence of astroglial FGF-2 immunoreactive nuclear profiles close to quies-

cent and reactive OX42 ir microglial profiles was quite common in the unlesioned tissue and particularly close to the 6-OHDA lesioned areas.

It is known that following the 6-OHDA injection in the ventral midbrain, the majority of the DA neurons disappears, in spite of the large amount of the endogenous astroglial FGF-2 synthesized (Chadi et al. 1994), a trophic factor with actions on DA neurons demonstrated *in vitro* (Ferrari et al. 1989; Park and Mytilineou 1992) and *in vivo* (Chadi et al. 1993a).

It also seems possible that the increased synthesis of the astroglial FGF-2 after the 6-OHDA-induced lesion of the ascending DA pathways might be related, exclusively, to the trophic events in the repair process (Chadi and Fuxe 1998). However, depending on the type and degree of the brain injury, the interaction of the reactive glial cells and the subsequent paracrine trophic actions on neurons might regulate *in vivo* the survival of the injured neurons (Giulian et al. 1993).

The interaction between reactive astrocytes and microglia in the 6-OHDA lesioned nigrostriatal DA pathways may be important in the balance of wound repair versus degeneration and thus for the local trophic regulation and subsequent neuronal protection and plasticity (Chadi and Gomide 2004, Gomide and Chadi 2005; do Carmo Cunha et al. 2007). In spite of several studies on this matter, there is a lack of information on the influence of adrenosteroid hormones on neurorestorative events mediated by glial cell interactions (Nichols et al. 2005). In fact, the ability of glucocorticoids to modulate glial reaction in the lesioned hippocampus is dependent on their effect in promoting local neuroprotection (Nichols et al. 2005).

The temporal analysis of the biochemical data of the Western blot of TH demonstrated that both ADX treatment and corticosterone replacement of adrenalectomized rats were able to modify the pattern of nigral 6-OHDA-induced decreases of TH level in the ventral midbrain. It seems that ADX with or without corticosterone replacement was able to potentiate the 6-OHDA-induced a time-dependent decrease of TH levels in the rat ventral midbrain. In fact, corticosterone treatment in 6-OHDA adrenalectomized rats promoted a further diminution of the TH levels in that region. These findings extend our previous observation regarding the inability of ADX to induce changes in that biochemical parameter in one time point (7 days) (Chadi et al. 2008). Further analysis is required to determine whether further diminution of TH levels in the ventral midbrain represents the ability of ADX to accelerate 6-OHDA-induced dopamine cell disappearance at the cellular level. The further time dependent decrease of TH levels in the midbrain of 6-OHDA/ADX/Cort group should also be highlighted, as an indication that the action of exogenous administration of corticosterone may potentiate dopamine lesion. These observations are in line with previous evidence of exogenous corticosterone administration inducing neuronal

lesion in regions of hippocampus (Dinkel et al. 2003). The possibility should be considered that exogenous, unlike endogenous regulated, glucocorticoids might trigger injury to neurons (Sapolsky and Pulsinelli 1985) and the injected corticosterone-induced increases of astroglial neurotrophic factor in the central nervous system may be related to a subsequent trophic response (Chadi et al. 1993b).

The effects of adrenocortical manipulation on midbrain TH levels were not reproducible in the striatum, which is probably due to a more rapid degeneration of DA terminals than the cell bodies (Chadi et al. 1994) thus requiring a more acute time-window of analysis.

These results should be taken into account when corticoid drugs are administered to patients with Parkinson's diseases, which may induce different effects on trophism of dopamine neurons to that triggered by the endogenous regulation of adrenocortical axis.

The adrenal steroids may act on neurons and glial cells through either glucocorticoid (GR) or mineralocorticoid receptors (Cintra et al. 1994b; Fuxe et al. 1994a, 1994b). A large number of central nervous system neurons and glia contain nuclear GR immunoreactivity like hippocampus (Cintra et al. 1994a, b; DeLeon et al. 1994). GR immunoreactivity was also found in astrocytes of the substantia nigra (Fuxe et al. 1994b) opening up the possibility of a adrenal steroid function on astroglial paracrine mechanisms in the basal ganglia.

The results of the GFAP levels in the ventral midbrain were partially coincident with those of TH levels in that region. No interaction treatment versus time effects were found among groups, probably because the changes in the levels of GFAP that have taken place in the small DA region were partially masked in the larger ventral midbrain region trimmed out for biochemical experiments. However, time-effects were observed that were in line with the results of adrenocortical manipulation in the midbrain TH levels. Remarkably, ADX seemed to induce higher GFAP levels in the 6-OHDA lesioned ventral midbrain of SHAM rats, which was further elevated with corticosterone replacement. The results are in line with the hypothesis that ADX potentiates the state of DA lesion-induced by nigral neurotoxin, which might be amplified with corticosterone replacement because the magnitude of astroglial reaction may be correlated to the degree of nervous tissue lesion. This correlation of adrenocortical manipulation triggering specific degree of DA lesion/astroglial activation may also have taken place in the striatum; however, only changes for differences were obtained probably due to a faster degenerative events in that region. Furthermore, we have not observed a substantial change of activation state of astrocytes labeled by immunohistochemistry after adrenalectomy (Chadi et al. 2008). Moreover, the results of this study cannot ensure that adrenocortical manipulation has interfered with the levels of

GFAP in reactive astrocytes or quiescent astrocytes, as described by Nichols and Finch (1994) or both. Furthermore, in the brain regions where neurons are dependent on adrenal steroids to survive, like hippocampal granule cells (Sloviter et al. 1993), the ADX seems to trigger a local increase of the GFAP positive reactive astrocytes (Gould et al. 1992).

These results of adrenocortical manipulation triggering differential changes in the lesioned DA pathways are in line with the present findings of biochemical analysis of FGF-2 levels in the ventral midbrain. The 23kDa isoform, which is expressed by astrocytes has diminished in that region after ADX. Considering the relation ipsilateral/contralateral side, corticosterone replacement was not able to counteract the ADX-induced diminution of FGF-2 levels in the ipsilateral lesioned side because an increased amount of protein could be found in the contralateral unlesioned side, as described by our group in a previous publication (Chadi et al. 2008). The current results are in full agreement with previous observations that have shown a downregulation of mRNA and protein of neurotrophic factors in normal or damaged brain after ADX (Barbany and Persson 1993; Sun et al. 1993; Chao and McEwen 1994; Riva et al. 1995a).

We have shown in the previous study that ADX reduced the increases of astroglial FGF-2 synthesis in the ipsilateral substantia nigra after a 6-OHDA-induced selective lesion of the nigrostriatal dopamine neurons without, however, interfering with the pattern of neuronal degeneration (Chadi et al. 2008). The influence of steroid hormones in astroglial FGF-2 mechanisms has been described in several brain regions (Riva et al. 1995b; Fuxe et al. 1994a).

An important feature of FGF mechanisms in the nervous system is the ability of FGF family members to mediate paracrine astroglial responses, particularly after injury, thus involved in neuroprotection and wound repair (Chadi and Gomide 2004; do Carmo Cunha et al. 2007). It was mentioned that adrenal glucocorticoids can modulate FGF mechanisms in astrocytes by interaction with steroid receptors (Magnaghi et al. 2000) and that astroglial FGF receptors can be modulated by glucocorticoid hormones (Riva et al. 1998). However there is a lack of information on the influence of adrenosteroid hormones in the neurorestorative events (Nichols et al. 2005). In fact, the ability of glucocorticoids to modulate glial reaction in the lesioned hippocampus is neuroprotection dependent (Nichols et al. 2005).

In this paper, employing a time-dependent modulation of DA lesion in the basal ganglia, we found a partial correlation involving the effects of adrenosteroid manipulation and the levels of TH, GFAP, and astroglial FGF-2. It seems that ADX may have favored DA lesion after 6-OHDA, and a subsequent astroglial reaction, by impairing the FGF-2-mediated trophic state in the basal ganglia. In view of the recent demonstrations of the presence of glucocorticoid receptors in microglia (Sierra et al. 2008) and the reduction the activation of

microglia and increased neuroprotection in lesioned spinal cord treated with glucocorticoid (Viscomi et al. 2008), the evaluation of the influence of adrenal steroid hormones on microglial reaction after DA lesion is still missing, which has been the subject of investigation of our laboratory.

In conclusion, reactive astroglia, but not reactive microglia, showed an increased FGF-2 IR in the process of DA cell degeneration induced by 6-OHDA. However, interactions between these glial cells may be relevant to the mechanisms that trigger the increased astroglial FGF-2 synthesis and thus may be related to the trophic state of DA neurons and the repair processes following DA lesion. Moreover, adrenocortical hormones may regulate astroglial-mediated trophic mechanisms and wound repair events in the lesioned DA system that may be relevant to the progression of Parkinson's disease. The characterization of the mechanisms related to the steroid regulation of the glial cell interaction-mediated wound repair and regeneration in the lesioned central nervous system are important to interpret the physiopathology of neural degeneration in diseases such as Parkinson's disease.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 16

Age and Parkinson's Disease-Related Neuronal Death in the Substantia Nigra Pars Compacta

Nina Eriksen, Anette Kirstine Stark, and Bente Pakkenberg

Abstract During aging, decline in memory and cognitive abilities as well as motor weakening is of great concern. The dopaminergic system mediates some aspects of manual dexterity, in addition to cognition and emotion, and may be especially vulnerable to aging. A common neurodegenerative disorder of this system, Parkinson's disease, is characterized by a selective, progressive loss of dopaminergic neurons in the substantia nigra pars compacta. This review includes studies quantifying age and Parkinson's-related changes of the substantia nigra, with emphasis on stereological studies performed in the substantia nigra pars compacta.

Keywords Aging • Dopaminergic neurons • Parkinson's disease • Substantia nigra pars compacta

Abbreviations list

MPTP	1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine
DA-ergic	Dopaminergic
GABA	Gamma-aminobutyric acid
VTApn	Paranigral part of VTA
PD	Parkinson's disease
SNpc	SN pars compacta
SNpr	SN pars reticularis
SN	Substantia nigra
SNCA	Synuclein alpha
TH+	Tyrosine hydroxylase-positive
VTA	Ventral tegmental area

Introduction

During aging, both morphological and neurochemical changes occur in the human central nervous system, which increase vulnerability toward physiological disturbances and neuropsychiatric disorders such as Parkinson's disease (PD) (see Morgan and Finch 1988). Many PD patients eventually develop cognitive decline (Aarsland et al. 2001). However, how brain function deteriorates during aging is still unclear, and whether cerebral physiology remains stable throughout middle age, followed by a rapid decline, remains unknown. The dopaminergic (DA-ergic) pathways are especially vulnerable to the effects of aging, and the neuropathology of PD is characterized by the progressive loss of substantia nigra (SN) DA-ergic neurons (McGeer et al. 1977; Severson et al. 1982; Carlsson 1987; Rinne 1987; Seeman et al. 1987; De Keyser et al. 1990; Kish et al. 1992; Rudow et al. 2007). In PD, regional differences in susceptibility to degeneration exist among midbrain DA neurons (Damier et al. 1999; Fearnley and Lees 1991; Gibb and Lees 1991). DA neurons in the ventral tier of the SN are most vulnerable to degeneration, while the dorsal tier of the SN and ventral tegmental area (VTA) are relatively resistant. Aging is the strongest risk factor for developing PD, and although marked age-related decline in the DA levels suggest that nigrostriatal system dysfunction occurs during normal aging (Volkow et al. 1996), its relationship with decline in cognitive function and age-related changes is unknown (see, e.g. Arranz et al. 1996). In some studies, regional patterns of age-related changes are similar to the patterns seen in PD (Irwin et al. 1994; Emborg et al. 1998; Gerhardt et al. 2002; McCormack et al. 2004; Chu and Kordower 2007; Kanaan et al. 2007, 2008). Despite the similarities between aging and PD, it remains unclear whether they are linked by shared cellular mechanisms of DA neuron vulnerability. This may have both vocational and clinical implications, because the DA-ergic system mediates some aspects of manual dexterity, in addition to cognition and emotion.

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The Nigrostriatal Pathway and PD

The nigrostriatal pathway runs from the SN pars compacta (SNpc) to the corpus striatum at the base of the cerebral hemisphere. Each nigral neuron forms more than a million varicosities (beads), which make synaptic contact with the dendrites of gamma-aminobutyric acid (GABA)-ergic neurons. The predominant effect of DA-ergic activity in the striatum is inhibitory, especially upon the enkephalin-containing GABA-ergic neurons projecting to the globus pallidus, whereas cholinergic neurons exert an excitatory effect. The SN pars reticularis (SNpr) is situated directly ventral to the SNpc and contains only purely GABA-ergic neurons. Some of them synapse in the superior colliculus and others in the mesencephalic reticular formation (for further details, see e.g. Katzung 1998). The mechanism by which the neurons in PD are lost may consist of an abnormal accumulation of the protein α -synuclein bound to ubiquitin in the damaged cells. This protein accumulation forms Lewy bodies (Spillantini et al. 1997, 1998). Some genes like synuclein alpha (SNCA) have been found to cause inheritable forms of PD, and gene products of SNCA are major components of Lewy bodies. Other PD inheritable genes (like UCHL1 and PARK2) are also involved in protein (de)ubiquitination. Therefore, dysregulated protein aggregation appears to be an important common factor in PD pathogenesis (Hol et al. 2005). Death of DA-ergic neurons by α -synuclein is due to a defect in the machinery that transports proteins between two major cellular organelles – the endoplasmic reticulum and the Golgi apparatus. Certain proteins like Rab1 (in yeast, *Saccharomyces cerevisiae*, Cooper et al. 2006; Gitler et al. 2008), and torsinA or TOR-2 (in the nematode, *Caenorhabditis elegans*, Cao et al. 2005) may reverse the defect caused by α -synuclein in experimental models. Bossers et al. (2008) found strong evidence for a reduction in neurotrophic support and alterations in axon guidance cues in the Parkinsonian SN, ultimately leading to DA-ergic cell death. Inflammation can also increase the risk of PD-like degeneration in DA-ergic neurons (Koprach et al., 2008). The loss of DA-ergic neurons in the SNpc results in reduced DA levels in the striatum, which in turn results in an increased inhibition of the ventral lateral nucleus of the thalamus, which sends excitatory projections to the motor cortex, thus leading to hypokinesia. This causes the majority of the clinical symptoms of PD (see Katzung 1998). Patients with severe neuronal loss in the SN generally also show pathology in the neocortex (Braak et al. 2003). The interaction of DA-ergic neurons with the surroundings might thus be important in neuronal vulnerability in PD (Damier et al. 1999).

Glial Responses

Another important component of PD pathogenesis is the glial response to DA neuron degeneration (Teismann and Schulz 2004). The administration of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) has shown regional differences in susceptibility to degeneration (Kitt et al. 1986; Schneider et al. 1987; German et al. 1988; Kanaan et al. 2007). In humans and other primates exposed to MPTP, reactive astrocytes are seen in the SN up to 16 years after the initial insult (Langston et al. 1999; Barcia et al. 2004). The biological significance of this astrocyte reaction is unclear. Using the optical fractionator, Kanaan et al. (2007) found no significant differences in the astrocyte number in the SN during aging in Rhesus monkeys, but more astrocytes were seen in the dorsal tier of the SN than the ventral tier and ventral tegment of the SN. The glial response, particularly that of microglia, and the influences of aging on these responses play an important role in the selective vulnerability of DA neurons (Kanaan et al. 2007).

We have reviewed studies quantifying age-related changes of the SN and provide tables of stereological investigations according to three species: rodents (Table 1), monkeys (Table 2), and humans (Table 3).

Age-Related Changes of the Nigrostriatal System

The first study to report a decrease in neuron numbers in the SN with age was carried out by McGeer et al. (1977) in a series of 28 persons without neurological disease, revealing a 48% cell loss by the age of 60, about 7% per decade. Although the methodological approach does not fulfill the criteria of recent cell-counting methods, this work remains an important point of reference supporting the concept of nigral neuronal loss with age. The stereological studies of the SN agree that there is a loss of neuromelanin-containing (pigmented) DA-ergic cells, in the SNpc during normal aging, both for rhesus monkeys (Pakkenberg et al. 1995; Emborg et al. 1998; Chen et al. 2000; Stark and Pakkenberg 2004) and humans (Ma et al. 1999a; Chen et al. 2000; Cabello et al. 2002; Chu et al. 2002; Stark and Pakkenberg 2004; Rudow et al. 2007). This also seems to be the tendency in other (nonstereological) histological studies of humans (e.g. Mann and Yates 1974; Mann and Yates 1979; Thiessen et al. 1990; Fearnley and Lees 1991; Tooyama et al. 1994). However, there is a disagreement on the rate of the loss of cells. Since PD is an age-associated disorder, it is important

Table 1 Stereological studies of the substantia nigra: rodents (*BDNF*: brain-derived neurotrophic factor, *CV* sd/mean, *mut* mutation, *NT* neurotrophic factor-3, *SN* substantia nigra, *SNpc* substantia nigra pars compacta, *SNpr* substantia nigra pars reticularis, *SURS* systematic uniform random sampling, *TH-ir* tyrosine-hydroxylase-immunoreactive neurons, *WT* wildtype). Modified from Stark and Pakkenberg (2004)

Species	Number (mean age)	Anatomy	Total number of neurons (CV)	Anatomical volume	Stereological design	Reference
Mouse, male	27 (12 weeks)	SN	Th-ir: 6,000–13,700		Optical disector, SURS Cryosections cut at 35 μ m Processed section thickness: ~16 μ m Disector height: 6 μ m Upper and lower guard zones Objective 100 $\times$ oil immersion	Chadi et al. (1993)
Rat	6 (28 days)	SNpc SNpr	Unilateral (right): 7,200 (0.15) 26,300 (0.07)		Optical disector, SURS Historesin sections cut at 40 μ m Processed section thickness 35 μ m Disector height 15 μ m Upper and lower guard zones Objective 100 $\times$ oil immersion	Oorschot (1996)
Rat, male	10 control 10 treated with BDNF 10 treated with NT	SNpr	Controls: 10,500 unilateral (left) BDNF: 10,000 unilateral (right) NT: 6,600 unilateral (right)	1.7 mm ³ 0.9 mm ³ 1.0 mm ³	Optical disector, SURS Cryosections cut at 40 μ m Processed section thickness and disector height not stated Upper guard zone: 5 μ m Objective 100 $\times$ oil immersion	Volpe et al. (1998)
Rat Mouse, male	6	SN SN	Th-ir: ~26,000 unilateral Non-pigmented: 43,000 unilateral		Semi-SURS, reporting only densities Optical disector, Cavalieri, SURS Cryosections cut at 40 μ m Processed section thickness: 21 μ m Disector height, guard zones and objective not stated	Canudas et al. (2000) Aguirre et al. (2001)
Mouse, male	16	Midbrain regions incl. SN	Th-ir: A8 (left): 1,461 A8 (right): 1,704		Cryosections cut at 50 μ m Processed section thickness: 20–21 μ m Disector height: 14–15 μ m Guard zones possible Objective 100 $\times$ oil immersion	Zaborszky and Vadasz, (2001)
Rat		SNpr			Optical disector, reporting only densities	Felberg et al. (2002)

to assess how the loss of neurons is associated with normal aging. In humans, brain weight, brain volume, and the total number of brain cells are greatest in the early teens, with no or only a small loss over the first five decades of adult life. During the age of 50–90 years, brain weight loss is approximately 2–3% per decade. Part of this weight loss comes from an increase in ventricular volume and the total amount of cerebrospinal fluid, but the precise location of the loss is difficult to pinpoint. Postmortem studies have found a significant reduction in the volume of the neocortex, archicortex, and white brain matter, whereas the volume of the central grey nuclei is not significantly reduced as a function of age (Esiri et al. 1997; Pakkenberg and Gundersen 1997; Stark and Pakkenberg 2004). Loss of neurons with aging has been reported in the neocortex, amygdala, spinal cord motor neurons, nucleus basalis, corpus striatum/putamen,

suprachiasmatic nucleus, inferior olive, and cerebellar Purkinje cells (Esiri et al. 1997). Primarily, the largest pigmented neurons in SN are lost in PD (Møller 1992). The loss of SN neurons has been estimated to be 4.3% (Cabello et al. 2002), and up to almost 10% per decade (Ma et al. 1999b) in humans during normal aging. Whether cerebral physiology remains relatively stable throughout middle age followed by a rapid period of decline or whether brain function deteriorates at a constant rate throughout adulthood is still unclear.

A few nonstereological studies have reported no cell loss in SN with age. Muthane et al. (1998) studied 84 brains from Indian people, aged 5–84 years, and found no decrease of SN neuron density with age. In 21 human control subjects aged 44–110 years, no significant loss of TH+ neurons in the SN was seen with increasing age (Kubis et al. 2000). In another investigation, Uchida et al. (2003) studied densities of TH+

Table 2 Stereological studies of the substantia nigra: monkeys (*CV* sd/mean, *DAT-ir* dopamine-transporter-immunoreactive neurons, *GTPC/IO-ir* GTP-cyclohydrolase-I-immunoreactive neurons, *MPTP* a neurotoxic drug, *SN* substantia nigra, *SNpc* substantia nigra pars compacta, *SNpr* substantia nigra pars reticularis, *SURS* systematic uniform random sampling, *α -syn-ir* α -synuclein-immunoreactive neurons, *TH-ir* tyrosine-hydroxylase-immunoreactive neurons). Modified from Stark and Pakkenberg (2004)

Species	Number (mean age)	Anatomy	Total bilateral number of neurons (CV)	Anatomical volume (CV)	Neuronal volume	Stereological design	Reference
Rhesus monkey		SN	Total numbers			Optical disector, Cavalieri, SURS Cut at 35 μ m	Pakkenberg et al. (1995)
<i>Macaca mulatta</i>	6 young females (8 years)		Young females: 320,000	68.9 mm ³ (0.24)			
	10 old females (23 years)		Old females: 312,000	62.8 mm ³ (0.28)		Disector height: 15 μ m	
	3 old alpha males (20 years)		Pigmented: Young females: 21,400 (1.13) Old females: 160,000 (0.52) Old males: 120,000 (0.31)			Processed section thickness and guard zones not stated Objective 100 $\times$ oil immersion	
			Non-pigmented: Young females: 139,000 (0.47) Old females: 285,000 (0.31) Old males: 130,000				
Rhesus monkey		SN	TH-ir:			Optical disector, Cavalieri, SURS Cryosections cut at 40 μ m	Emborg et al. (1998)
<i>Macaca mulatto</i>	7 young males (3–5 years)		Young: 111,000	54 mm ³		Processed sections thickness: 38 μ m	
	4 old males (26–28 years)		Old: 55,000	48 mm ³		Disector height 25 μ m	
	3 old females (26–28 years)		DAT-ir: Young: 107,000 Old: 71,000			Upper guard zone 1–2 μ m, lower guard zone possible within 38 Objective 60 $\times$ Planapo oil immersion	
Green monkey <i>Ceropithecus aethiops</i> <i>sahaeus</i>	1 control (4 years) 2 hemisphere- tomized (4 years)	SN	Unilateral: ~320,000 SNpc: ~230,000 (0.20)			Optical disector, Cryosections cut at 40 μ m Disector height: 10 and 15 μ m Processed section thickness and guard zones not stated Objective 100 $\times$	Theoret et al. (1999)
Rhesus monkey	6 young males (4–5 years)	SN	GTPCHI.ir unilateral (left):			Processed section thickness: 28 μ m	Chen et al. (2000)
<i>Macaca mulatto</i>	2 old males (23–27 years)		Young: 206,000	50 mm ³	6,600 μ m ³	Disector height: 20 μ m	
	4 old females (23–27 years)		Old: 67,000	42 mm ³	4,100 μ m ³	Upper guard zone: 5 μ m, lower guard zone: 2–3 μ m Objective 100 $\times$ Planapo oil immersion	

Rhesus monkey		SNpc	TH-ir, unilateral (right):		Optical fractionator	Gerhardt et al. (2002)
<i>Macaca mulatto</i> , females	4 young (8–9 years)		Young: 230,000	~380 μm^3	Cryosections cut at 40 μm	
	5 mid-aged (14–17)		Mid-aged: ~190,000		Disector height: 20 μm	
	4 old (23–28 years)		Old: 170,000		Processed section thickness, guard zones and objectives not stated	
Rhesus monkey	8 males and 16 females (2–34 years)	SN	Pigmented:		No description of cell size estimation methods	
<i>Macaca mulatto</i>	4 young (2–12 years)		Young: 17,656		Optical disector, SURS	Chu and Kordower (2007)
	10 mid-aged (15–24 years)		Mid-aged: 47,604		Cryosections cut at 40 μm	
	10 old (24–34 years)		Old: 55,683		Processed section thickness: 26 μm	
			α -syn-ir:		Disector height: 11 μm	
			Young: 359.07		Guard zones: 3 μm	
			Mid-aged: 525.94		Objective 100 $\times$ Planapo oil immersion	
			Old: 657.38			

neurons, glialacidic protein, neurofilaments, and single-stranded DNA in the SN of dogs, and found no significant age-related decrease in neuronal density.

SN Neuronal Volume

There seems to be some disagreement as to what happens to the volume of the SN neurons with age. In an early nonstereological study by Mann and Yates (1979), of 70 men and women from newborn to 91 years, a 20% decrease in SN neuronal nucleus volume was reported. Others have also found a decrease in nucleus volume in neurons of the SNpc during normal aging (Finch 1993). In their Golgi study, Cruz-Sánchez et al. (1995) described distorted profiles of the cell body, loss of dendrites and dendritic spines, and swelling and beading of the dendritic branches as common changes in neurons from the oldest group (70–93 years). Some neuronal bodies were enlarged and some dendrites showed a tortuous profile. Finch (1993) has suggested that the accumulation of melanin and lipofuscin, and the presence of vacuoles are strongly related to the displacement of cell cytoplasm considered to be localized, age-related changes. In metabolic disorders, neurons show swelling of perikarya and torpedo-like axonal and dendritic swellings that have been associated with the progressive accumulation of uncatabolized substrates (Braak and Goebel 1979; Jagadha and Becker 1988). The reductions in cell volume

are not in agreement with the findings of Cabello et al. (2002), who, using the rotator method, found an increase in the volume of these neurons as a function of age, as did Rudow et al. (2007) using the optical disector (for specific numbers see Table 3). Cabello et al. (2002) also employed a combination of the total number of melaninpositive neurons and their size distribution and calculated the total perikaryon volume of melanin-positive neurons; this showed no decrease with increasing age. The stereological results are in agreement with Møller (1992). In a stereological study of 26 individuals aged 0–85 years, Ma et al. (1999b) found that the area of the neuronal cell body decreased by 3.2% per decade, which is in contrast to the finding by Cabello et al. (2002). Ma et al. (1999b) do, however, point out that measurements of cell areas in two-dimensional sections do not necessarily correspond to a decrease in cell volume. Furthermore, the area measurements were performed on paraffin sections in contrast to the plastic sections used by Cabello et al. (2002). Both Cabello et al. (2002) and Møller (1992) included all pigmented neurons in the entire SN, whereas Ma et al. (1997), Ma et al., 1999a) performed their measurements on the SNpc only.

Siddiqi et al. (1999) studied the relationship between age and the overall SN volume plus the number and volume of neurons in the SNpc and in the ventral tegmental area (VTA) in 14 rhesus monkeys. They found no significant relationship between age and the overall volume of these DA-ergic brain stem nuclei, but there was a significant loss of the total number of neurons in the SNpc and the paranigral part of

Table 3 Stereological studies of the substantia nigra: humans (AD: patients with Alzheimers disease, CV = sd/mean, DAT-ir: dopamine-transporter-immunoreactive neurons, GTPC/IO-ir: GTP-cyclohydrolase-1-immunoreactive neurons, PD: patients with Parkinson's disease, PDD: patients with Parkinson's disease, SNpc: substantia nigra pars compacta, SNpr: substantia nigra pars reticularis, SURS: systematic uniform random sampling, α -syn-ir: α -synuclein-immunoreactive neurons, TH-ir: tyrosine-hydroxylase-immunoreactive neurons), Modified from Stark & Pakkenberg (2004)

Species	Number (mean age)	Anatomy	Total bilateral number of neurons (CV)	Anatomical volume (CV), mm ³	Neuronal volume (CV), μ m ³	Stereological design	Reference
Human <i>Homo sapiens</i>	7 controls (78 years) 7 PD (83 years): 4 women, 3 men	SN	Pigmented: Controls: 550,000 Non-pigmented: Control: 260,000	Controls: 256 PD: 190		Physical disector, Cavalieri Paraffin sections cut at 11 μ m Processed section thickness: 6.3 μ m Objectives 20 $\times$ Cell counting and volume estimation performed at the same processing level	Pakkenberg et al. 1991
Human <i>Homo sapiens</i>	3 PDD (75 years): 2 women, 1 man 3 Controls (73 years)	SN			PDD: 28: 900 Controls, 31: 300	Rotator, SURS Plastic sections cut at 35 μ m Processed section thickness not stated (usually no shrinkage in plastic) Disector height and guard zones not stated	Møller 1992
Human <i>Homo sapiens</i>	6 young (27 years) men 5 mid-aged (58 years): 3 women + 2 men 5 aged (80 years): 2 women + 3 men	SN	DAT-ir: Young: 103,000 Mid-aged: 67,000 Aged: 61,000	47 37 37		Optical disector, Cavalieri, SURS Cryosections cut at 40 μ m Processed section thickness 34–44 μ m Disector height: possibly 6 $\times$ 5 μ m Upper guard zone: 1–2 μ m, plus lower guard zone	Ma et al. 1999a
Human <i>Homo sapiens</i>	9 (18–85 years): 3 young (26 years) 3 mid-aged (58 years) 3 aged (85 years)	Mid brain part of SN	GTPCHI-ir: Young: 142,000 Mid-aged: 59,000 Aged: 26,000	70 77 72	8,700 9,800 9,400	Objective 60 $\times$ Planapo oil immersion Disector height 20 μ m Processed section thickness and guard zones not stated	Chen et al. 2000
Human <i>Homo sapiens</i>	4 controls 5 PDD (65–80 years)	SNpc	TH-ir: Controls: 250,000 PDD: 12,000 – 66,000			Objective 100 $\times$ Planapo oil immersion Optical fractionator, SURS Cryosections cut at 50 μ m Processed section thickness: 20 μ m Disector height: 10 μ m Guard zones sufficient Objective 40 $\times$	Perl et al. 2000
Human <i>Homo sapiens</i>	28 men (19–92 years): 7 young (27 years) 14 mid-aged (57 years) 7 aged (82 years)	SN	Pigmented: 320,000 Non-pigmented: 64,000 Young: 380,000 Aged: 260,000	165 138	21,000 52,000	Optical disector, Cavalieri, rotator, SURS Plastic sections cut at 40 μ m Processed section thickness not stated (usually no shrinkage in plastic) Disector height: 15 μ m	Cabello et al. 2002
Human <i>Homo sapiens</i>	6 young (29 years) 6 mid-aged (57 years) 7 aged (87 years)	SNpc	TH-ir: Young: 214,000 Mid-aged: 190,000 Aged: 115,000 Nurr-1-r: Young: 206,000 Mid-aged: 158,000 Aged: 111,000	93 93 84		Cryosections cut at 40 μ m Processed section thickness: 26 μ m Disector height: 15 μ m Upper guard zone 5 μ m, lower guard zone > 4 μ m Objective 100 $\times$ Planapo oil immersion	Chu et al. 2002
Human <i>Homo sapiens</i>	11 AD (84 years): 7 women, 4 men	SNpc	Unilateral (right) pigmented	167 181		Physical disector, Cavalieri Paraffin sections cut at 11 μ m Cell counting and volume estimation	Kemppainen et al. 2002

Although age-related cellular hypertrophy is usually considered an indication of cell degeneration or necrosis; this may not always be the case. Despite overall loss of neurons over time, clinical cognitive and motor deficits in the elderly are of a limited nature (Stanford et al. 2003). Rudow et al. (2007) found significant hypertrophy in pigmented and TH+ neurons in older controls compared with younger controls, in contrast to a significant atrophy in PD patients. This suggests a compensatory mechanism within individual DA-producing SN neurons that facilitates normal motor function despite the loss of neurons in normal aging. During PD, this compensatory mechanism is possibly degenerated, leading to the onset of motor disturbance (Rudow et al. 2007). Thus, the total amount of cell substance capable of producing the essential transmitters may not be reduced to a critically low level as a result of aging, and hence, the amount of transmitter per cell may also be increased (Faraldi et al. 1994; Cantuti-Castelvetri et al. 2003). As pointed out by Ma et al. (1999a), neuronal changes can be highly selective during aging. The reason for the age-related cell loss is not self-evident, although several hypotheses have been proposed, such as the

presence of a mitotic clock, programmed cell death (Anglade et al. 1997), the expression of specific genes, or oxidative stress (Kish et al. 1992).

Oxidative Stress Hypothesis

Vulnerability of the DA-ergic neurons is considered to be a result of oxidative stress caused by the increased generation of reactive oxygen species with aging (Giovannelli et al. 2003), the reduced capacity of the antioxidant system (Itoh et al. 1996; Naoi and Maruyama 1999), and changes in the microglial activation pattern (Sugama et al. 2003). However, Kish et al. (1992) found no relationship of striatal areas with higher DA content and striatal areas vulnerable to PD. Thus, they could not conclude that the oxidative stress hypothesis is an explanation for DA-ergic cell loss. In a stereological study by Zhao et al. (2003) neurogenesis was found in SNpc in adult mice. The nigrostriatal degeneration that occurs in normal aging may be associated with mild “Parkinsonian” signs, but PD, unlike aging, involves a mechanism producing a regionally specific (putamen) DA-ergic loss (Gibb and Lees 1991; Kish et al. 1992).

Increasing α -synuclein in the Elderly

Chu and Kordower (2007) found a robust association between α -synuclein and aging within nigral neurons in both humans and nonhuman primates, with a statistically significant larger aggregation of α -synuclein with age. α -synuclein was almost undetectable within nigral perikarya in young monkeys and humans as α -synuclein immunoreactivity in these cases was restricted to fibers and terminals. The increase of neurons expressing immunoreactive to α -synuclein-ir was very high in middle aged and aged compared with young cohorts. However, these age-related α -synuclein-ir expressions are not in the form of inclusion as seen in PD.

DA Receptors and Aging

Aged rhesus monkeys (25–27 years old), compared with young rhesus monkeys (3–5 years old), have a significantly impaired motor function accompanied by an age-related decrease of SN immunoreactivity for TH+ and the DA transporter (van Dyck et al. 1995; Emborg et al. 1998), which is considered a reliable marker of presynaptic DA-ergic terminal loss (Miller et al. 1997). A decrease in striatal DA D₁- (Suhara et al. 1991; Nabeshima et al. 1994; Wang

et al. 1998) and D₂- receptors (Morgan et al. 1987; Antonini and Leenders 1993; Rinne et al. 1993; Mescio et al. 1993) and a loss of binding of DA transporter ligands have been reported with age (Cross et al. 1984; Lai et al. 1987; Rinne 1987; Seeman et al. 1987; Rinne et al. 1990; Bannon et al. 1992; Himi et al. 1995; Volkow et al. 1996; Ma et al. 1999a). D₁- and D₂- receptor density is not decreased directly in SN (De Keyser et al. 1990, 1991), but a decrease in DA-ergic uptake sites of the putamen (De Keyser et al. 1990) and a decrease in postmortem brain DA content with age have been observed (Carlsson and Winblad 1976). Other research papers have reported no change in striatal dopamine levels with age, but an increase of DA content in the cerebral cortex (Godefroy et al. 1989). A review of the receptor density, affinity, and functional capacity through functional imaging has been presented by Reeves et al. (2002), as well as several reviews on DA-ergic changes with age given by Kaasinen and Rinne (2002), Luo and Roth (2000), Morgan and Finch (1988), and Wong et al. (1988).

Concluding Remarks

We have reviewed current as well as historical evidence for age-related, DA-ergic cellular changes in the nigrostriatal system. There seems to be a general agreement that the number of neurons in the SN decreases with age and especially in patients suffering from PD. However, there is no agreement about the size of this decrease, either in normal aging or in PD. Prior to the decrease in neuron numbers in the SN during aging, some investigations have shown an increase in neuronal volume, possibly explaining why these healthy elderly can withhold their DA levels. In PD, this neuronal volume increase is not seen. Thus, the ability to maintain a normal DA level fails, and the symptoms of PD arise.

Increasing knowledge of the cellular substrate of the nigrostriatal system, the availability of more specific drugs acting on these cells, and evaluation of the influence of age-related neurological and psychiatric disorders on the functions of these cells will provide a better basis for evaluating the role of the DA-ergic system in health and disease.

Some studies show that anti-inflammatory drugs may be one of the new approaches in the treatment of PD. Extracellular neuromelanin could be one of the key molecules leading to microglial activation and neuronal cell death in the SN. This may be highly relevant to the elucidation of therapeutic strategies in PD (Wilms et al. 2007; Zecca et al. 2008). Gao et al. (2008) demonstrated a neuroprotective effect of triptolide on dopaminergic neurons in 1-methyl-4-phenyl pyridinium (MPP+)-induced hemi-Parkinsonian rats. They concluded that the protective effect of triptolide may

be related to the inhibition of MPP(+)-induced microglial activation, and support the use of immunosuppressive agents in the management of PD.

As a large number of investigations have reported only density measurements, further work will be required to assess possible specific cell changes in the normally aged nigrostriatal system and in the PD nigrostriatal system. Also of importance for future studies in this area will be evaluation of whether alterations of nigrostriatal DA-ergic neurotransmission represent a cause or merely a consequence of other neurotransmitter system changes occurring during physiological and pathological aging.

Conflicts of Interest Statement We declare that we have no conflict of interest.

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Chapter 17

Neurodegeneration in Parkinson's Disease: Genetics Enlightens Physiopathology

Olga Corti, Margot Fournier, and Alexis Brice

Abstract Parkinson's disease (PD) is a severe neurodegenerative disorder of complex etiology and enigmatic physiopathology. In the past decade, the identification of genes involved in rare familial Parkinsonian syndromes has brought hope that understanding the functions of their products will provide insight into the molecular mechanisms responsible for neurodegeneration. The knowledge accumulated thus far has delineated two putative, potentially interconnected, disease-causing pathways: α -synuclein accumulation may be central to Parkinsonism due to α -synuclein gene defects, but possibly also to sporadic PD and other genetic forms presenting with Lewy bodies; altered mitochondrial physiology may be pivotal to Parkinsonian syndromes caused by parkin, PINK1, and possibly DJ-1 gene mutations. Adding new pieces to this fragmentary picture to determine to what extent sporadic PD and Parkinsonism due to distinct genetic causes share common pathogenic mechanisms remains a major challenge toward the development of future therapeutic strategies for these disabling disorders.

Keywords DJ-1 • Lewy bodies • Parkin • Parkinson's disease • PINK1 • α -synuclein

Abbreviations

LBs Lewy bodies
PD Parkinson's disease
SNc Substantia nigra pars compacta

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Introduction

Parkinson's disease (PD) is a common, devastating neurodegenerative disorder characterized by a triad of motor symptoms – bradykinesia, rigidity, and resting tremor, – caused by the preferential degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc). Intra-neuronal inclusions, mainly composed of α -synuclein and ubiquitin, termed Lewy bodies (LBs), are invariably associated with the sporadic form of the disease. Although the etiology of PD remains poorly understood, environmental risk factors and genetic predisposition are believed to contribute to its development. In the past decade, the identification of genes causing rare familial Parkinsonian syndromes has shed new light on the pathophysiology of this complex disorder. Approximately 10% of the patients with typical PD have a family history compatible with Mendelian inheritance. Twelve loci have been linked to monogenic forms of PD, and genetic data unequivocally support the involvement of seven genes in these disorders (Table 1). Mutations in α -synuclein, *LRRK2*, and the most recently identified *GIGYF2* gene are transmitted with an autosomal dominant mode of inheritance and are thought to confer a toxic function on the encoded proteins; mutations in *parkin*, *PINK1*, *DJ-1*, and *ATP13A2* are autosomal recessive and believed to lead to the loss of protein function. It is suspected that other genes may be altered in familial PD, including *UCH-L1* and *HtrA2/Omi*, but the genetic evidence for their involvement is poor (for review, see Biskup et al. 2008).

Are Nigral Dopaminergic Neurons Particularly Vulnerable to Proteotoxic Stress?

The idea that altered protein handling plays a role in the pathogenesis of PD has gained strength with the discovery of the E3 ubiquitin-protein ligase activity of Parkin, encoded by the gene most commonly mutated in early-onset Parkinsonism

Table 1 Loci and genes involved in familial parkinsonian syndromes and their suspected functions

Locus	Gene	Inheritance	Disease characteristics: <i>Onset/Progression/LBs</i>	Protein function
PARK1/4	α -synuclein	AD	Variable/Severe/Yes	Maintenance of synaptic vesicle pool, vesicle priming; multifunctional
PARK2	parkin	AR	Early/Slow/Variable	Neuroprotective E3 ubiquitin protein ligase; multifunctional
PARK3	?	AD	Late/Slow/Yes	?
PARK5	UCH-L1?	AD?	Early/?/?	Ubiquitin hydrolase, ubiquitin ligase
PARK6	PINK1	AR	Early/Slow/?	Neuroprotective mitochondrial serine-/threonine kinase; multifunctional
PARK7	DJ-1	AR	Early/Slow/?	Oxidative stress sensing; multifunctional
PARK8	LRRK2	AD	Variable/?/Variable	Neurite morphology, signalling; multifunctional kinase
PARK9	ATP13A2	AR	Juvenile/Severe/?	Putative lysosomal type 5 P-type ATPase
PARK10	?	AD?	Late/?/?	?
PARK11	GIGYF2	AD	Late/Typical/?	Insulin-like growth factor / Insulin signalling?
PARK12	?	Susceptibility gene ?	Late/?/?	?
PARK13	Omi/HtrA2	Susceptibility gene?	Late/?/?	Mitochondrial protease, antiapoptotic functions

with autosomal recessive inheritance (for review, see Corti and Brice 2007). The function of Parkin was intuitively linked to that of the proteasome, and its loss in *parkin*-related PD was thought to lead to the accumulation of undegradable, toxic substrates. However, whether the pharmacological inhibition of proteasome activity triggers selective dopaminergic neuron degeneration in vivo is controversial (McNaught et al. 2004; Bove et al. 2006; Kordower et al. 2006; Schapira et al. 2006). Despite demonstrations of the toxicity of potential Parkin substrates in cell or animal models (Imai et al. 2001; Corti et al. 2003; Dong et al. 2003; Yang et al. 2003; Kitao et al. 2007), there is also debate as to whether Parkin deficiency leads to their accumulation in knockout animals and in *parkin*-related PD patients. Proteasomal targeting is only one of several vocations of ubiquitylation processes, and Parkin may not generally be involved in substrate degradation (Lim et al. 2005; Fallon et al. 2006; Hampe et al. 2006; Matsuda et al. 2006; Henn et al. 2007).

It is unclear whether Parkin regulates α -synuclein homeostasis. The molecular mechanisms involved in α -synuclein clearance are a matter of debate, and it is likely that both ubiquitin-dependent and -independent proteasomal degradation and autophagy are involved, perhaps acting on different α -synuclein conformers (Bennett et al. 1999; Ancolio et al. 2000; Rideout et al. 2001; Tofaris et al. 2001; Webb et al. 2003; Cuervo et al. 2004; Lee et al. 2004; Rideout et al. 2004). Parkin and α -synuclein do not interact according to an enzyme–substrate relationship. When overproduced, Parkin protects against α -synucleinopathy in various models (Petrucelli et al. 2002; Yang et al. 2003; Haywood and Staveley 2004), but its deficiency does not exacerbate the deleterious effects of α -synuclein in transgenic mice (von Coelln et al. 2006). How this relates to *parkin*-related or sporadic PD is unclear. Typical LBs are only rarely observed

in PD patients carrying *parkin* gene mutations (for review, see Cookson et al. 2008), which could either point to a disease entity that is not an α -synucleinopathy, or on the contrary, could suggest that Parkin promotes the accumulation of fibrillar α -synuclein in LBs.

Whether LBs are a requirement for the definite diagnosis of PD is increasingly being challenged. Clinically typical PD patients may show nigral dopaminergic neuron degeneration in the absence of LBs; in members of one and the same family with PD linked to a single mutation in LRRK2, neurodegeneration may occur alone, with typical brainstem-type LBs, with cortical LBs, or with tauopathy (reviewed by Cookson et al. 2008). The significance of protein deposits – a sign of primary pathology or a mere epiphenomenon – remains unclear. Several findings suggest that the process of α -synuclein aggregation may be central to the disease, although there is debate over whether the final product of this process, the LB, or precursor species, are deleterious (reviewed by Shults 2006). The overdosage of normal α -synuclein leads to the formation of inclusions and to neurodegeneration in animal models (Feany and Bender 2000; Kirik et al. 2002; Eslamboli et al. 2007) and in patients with genomic multiplications of the α -synuclein gene, who develop PD in the presence of LBs (Singleton et al. 2003; Chartier-Harlin et al. 2004; Ibanez et al. 2004; Cookson et al. 2008). The three known PD-causing missense mutations increase the propensity of the protein to form soluble protofibrils in vitro (Conway et al. 2000a, 2000b; Fredenburg et al. 2007), suggesting that these unstable oligomeric intermediates are the disease-causing molecular species. Consistent with this possibility, dopamine has been reported to covalently modify and stabilize α -synuclein protofibrils, which might explain why dopaminergic neurons are particularly vulnerable to α -synuclein-mediated toxicity (Conway et al. 2001). Protofibrils might embed in biological

membranes, leading to the formation of permeabilizing nanopore like structures, although whether this happens in vivo remains to be demonstrated (Volles and Lansbury 2002). There is evidence that accumulating α -synuclein may interfere with protein degradation pathways, which may in turn accelerate its buildup: PD-causing variants of α -synuclein impair proteasomal activity in cell models (Stefanis et al. 2001; Petrucelli et al. 2002) and compromise the lysosomal uptake and the degradation of substrates of chaperone-mediated autophagy, including α -synuclein (Cuervo et al. 2004). The recently discovered, unexpectedly close relationship between PD and the autosomal recessive lysosomal storage disorder, Gaucher disease, raises the possibility that defects in the lysosomal proteolytic pathways are involved in the pathogenesis of PD: Gaucher disease is an autosomal dominant disorder due to mutations in the gene encoding glucocerebrosidase leading to an intracellular buildup of glucosylceramide; in the heterozygous state, these mutations greatly increase the risk of PD (Gan-Or et al. 2008; Rogaeva and Hardy 2008). Finally, a causative role for lysosomal dysfunction in PD has been suggested by the recent identification of mutations in the ATP13A2 gene, encoding the lysosomal type 5 P-type ATPase, as responsible for familial Parkinsonism (Ramirez et al. 2006; Di Fonzo et al. 2007).

Do Mitochondrial Dysfunction and Oxidative Stress put Nigral Dopaminergic Neurons at Risk?

It has long been suspected that chronic exposure to mitochondrial complex I inhibitors could contribute to the development of sporadic PD, and the possibility that mitochondrial DNA mutations account for the moderate dysfunction of this complex in this disease is still a matter of debate (for review, see Fukui and Moraes 2008). Three out of the four known genes responsible for autosomal recessive Parkinsonism, *parkin*, *DJ-1*, and *PINK1*, have been linked to mitochondrial functions (reviewed by Dodson and Guo 2007). A fraction of Parkin is associated with the outer mitochondrial membrane (Darios et al. 2003); DJ-1 has been associated with the outer membrane and the matrix (Canet-Aviles et al. 2004; Zhang et al. 2005); the serine/threonine kinase PINK1 is synthesized as a precursor with an N-terminal mitochondrial targeting sequence (Valente et al. 2004). The three proteins have pleiotropic neuroprotective properties in a number of cell-death paradigms, involving mitochondrial dysfunction or oxidative stress (reviewed by Alves da Costa 2007; Corti and Brice 2007; Mills et al. 2008). There is evidence of mitochondrial dysfunction and oxidative damage in *parkin* and *PINK1*

knockout mice, as well as in PD patients with mutations in these genes (Muftuoglu et al. 2004; Palacino et al. 2004; Hoepken et al. 2007; Gautier et al. 2008). Elegant genetic studies in *Drosophila* have suggested that Parkin and PINK1 may act in a common biochemical pathway centred on the maintenance of mitochondrial functions. Invalidating the *Drosophila* orthologs of either *parkin* or *PINK1* leads to strikingly similar phenotypes, including male sterility and flight muscle degeneration, preceded by severe mitochondrial pathology (Greene et al. 2003; Clark et al. 2006; Park et al. 2006; Yang et al. 2006). These phenotypes can be suppressed by the overproduction of Parkin but not PINK1, indicating that Parkin acts upstream of PINK1 in a potentially linear pathway. Similarly, alterations in mitochondrial morphology caused by PINK1 deficiency in human cells are rescued by overproduced Parkin (Exner et al. 2007). Strikingly, loss-of-function mutations, or increased dosage of genes encoding key components of the mitochondrial fusion/fission machinery, severely modify these mitochondrial phenotypes in *Drosophila*, providing compelling evidence that the PINK1/Parkin pathway modulates mitochondrial morphology (Deng et al. 2008; Poole et al. 2008; Yang et al. 2008). Despite these remarkable advances in research on flies, these observations have not been confirmed in mouse models thus far, and it remains to be determined how far the highlighted mechanisms underlie nigral dopaminergic neurodegeneration in *parkin*- and *PINK1*-related Parkinsonism.

Mechanisms of Dopaminergic Neurodegeneration in Parkinson's Disease: Multiple Routes to a Unique Solution?

There is great hope that progress in our understanding of the function of the genes involved in inherited Parkinsonian syndromes will shed light on the physiopathology of sporadic PD. This hope is based on the idea that there are shared pathogenic mechanisms in these disorders. It is supported by the knowledge acquired on other neurodegenerative diseases, particularly Alzheimer's disease, most often sporadic, but sometimes caused by the alteration of genes involved in amyloid precursor protein metabolism. By comparison, the evidence that the known PD genes are parts of a single puzzle is poor. Efforts have been made to discover direct relationships between the protein products of these genes, but despite some success, the physiological relevance of the identified interactions remains elusive. Accumulation of α -synuclein most likely defines a group of etiologically related diseases, linking the most common sporadic PD at least, to inherited Parkinsonism induced by α -synuclein gene alterations. It is unclear as to how far Parkinsonism due to mutations in the other PD genes is related to α -synucleinopathy:

LBs are observed in some patients with *LRKK2* or *parkin* gene mutations, but they are absent in others and the neuropathological features of the diseases caused by other gene alterations are still unknown (Cookson et al. 2008). In principle, even the occasional presence of these inclusions may hint at the involvement of α -synuclein. Different conformers could accumulate, depending on the interaction with other intrinsic or extrinsic factors. Whether mitochondrial dysfunction contributes to the buildup of α -synuclein species is uncertain: LBs are not observed in MPTP-induced Parkinsonism in humans (Langston et al. 1999), and conflicting results have been reported in animals (Fornai et al. 2005; Alvarez-Fischer et al. 2008). However, α -synuclein could amplify the toxicity of complex I inhibitors (Dauer et al. 2002; Schluter et al. 2003; Klivenyi et al. 2006), and its overexpression may contribute to mitochondrial dysfunction (Hsu et al. 2000; Martin et al. 2006; Stichel et al. 2007; Devi et al. 2008). It is suspected that similar interaction loops exist between mitochondrial dysfunction, ATP-dependent protein degradation pathways, and α -synuclein buildup. Studies in invertebrate models have provided evidence that pathways long suspected to intervene in the physiopathology of sporadic PD modify not only the phenotype caused by α -synuclein overexpression but also that induced by the alteration of other PD-related proteins. For example, genetic or pharmacological manipulation of pathways involved in glutathione synthesis or conjugation enhanced α -synuclein toxicity in yeast (Willingham et al. 2003), exacerbated dopamine neuron loss in a *Drosophila* model of α -synucleinopathy (Trinh et al. 2008) and in *parkin* mutant flies (Whitworth et al. 2005), and decreased viability in DJ-1 knockout flies (van der Brug et al. 2008). Compensatory changes in glutathione levels and metabolism have been reported in *parkin* knockout mice (Itier et al. 2003), which show only moderate dopaminergic phenotypes, indicating that this small molecule may well be ‘the elephant in the room’, as recently suggested (Zeevalk et al. 2008). Therefore, it is theoretically possible that diseases with apparently distant etiologies share common pathological processes.

Conclusions

Spectacular advances in the genetics of PD in the past decade have shed light on some of the molecular pathways that could be involved in the pathogenesis of familial Parkinsonian syndromes. Some parallels have emerged between the mechanisms suspected of intervening in these disorders and the more frequent sporadic disease. Two major, possibly interconnected pathways, centred on α -synuclein accumulation and mitochondrial physiology, have emerged. Further studies in cell and animal models are essential to fill in the

gaps in our knowledge, and determine whether the already available fragments of information define a single, rather than several independent physiopathological, puzzles. Major as-yet-unresolved issues that the scientific community will have to address further concern the molecular basis of the selectivity of the lesions in PD: why do specific neuronal populations, particularly the dopaminergic neurons of the SNc, die preferentially? what specific defenses did they develop to protect themselves from intrinsic threats, e.g. massive activity-dependent Ca^{2+} influx or cytosolic dopamine-related oxidative stress? why are other dopaminergic neurons relatively spared? where do the products of the PD-related genes meet the specificities of the vulnerable neurons? Answering these questions should provide clues to the physiopathology of this complex disorder and open new therapeutic perspectives.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 18

In Vivo Microdialysis in Parkinson's Research

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Abstract Parkinson's disease (PD) is a progressive neurodegenerative disorder that is primarily characterized by the degeneration of dopamine (DA) neurons in the nigrostriatal system, which in turn produces profound neurochemical changes within the basal ganglia, representing the neural substrate for parkinsonian motor symptoms. The pathogenesis of the disease is still not completely understood, but environmental and genetic factors are thought to play important roles. Research into the pathogenesis and the development of new therapeutic intervention strategies that will slow or stop the progression of the disease in human has rapidly advanced by the use of neurotoxins that specifically target DA neurons. Over the years, a broad variety of experimental models of the disease has been developed and applied in diverse animal species. The two most common toxin models used employ 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/1-methyl-4-phenylpyridinium ion (MPTP/MPP⁺), either given systemically or locally applied into the nigrostriatal pathway, to resemble PD features in animals. Both neurotoxins selectively and rapidly destroy catecholaminergic neurons, although with different mechanisms. Since in vivo microdialysis coupled to high-performance liquid chromatography is an established technique for studying physiological, pharmacological, and pathological changes of a wide range of low molecular weight substances in the brain extracellular fluid, here we review the most prominent animal and human data obtained by the use of this technique in PD research.

Keywords 6-OHDA • Basal ganglia • Free oxygen radicals • Microdialysis • MPP⁺ • MPTP • Parkinson's disease • ROS

Abbreviations

·OH	Hydroxyl radicals
¹ O ₂	singlet oxygen
2-HBA	Salicylate
4-HBA	4-hydroxybenzoic acid
5-HIAA	5-hydroxyindoleacetic acid
6-OHDA	6-hydroxydopamine
7-NI	7-Nitroindazole
A _{2A}	Adenosine2A
ACE	Angiotensin II converting enzyme
BDNF	Brain-derived neurotrophic factor
COX	Cyclooxygenase
D ₃	calcitriol
DA	Dopamine
DAT	DA transporter
DBS	Deep brain stimulation
DOPAC	3,4-dihydroxyphenyl acetic acid
ESC	Embryonic stem cells
GDNF	Glial cell line-derived neurotrophic factor
GLU	Glutamate
GPe	Globus pallidus external segment
GPi	Globus pallidus internal segment
GSH	Glutathione
GSSG	Glutathione disulfide
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
L-DOPA	L-3,4-dihydroxyphenylalanine
L-NAME	N (G)-nitro-L- arginine methyl ester
MAO	Monoamine oxidases
MPP ⁺	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSC	mesenchymal stem cells

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NMDA	N-methyl-D-aspartate
nNOS	neuronal Nitric oxide synthase
NOP	Nociceptin/orphanin FQ
O ₂ ⁻	Superoxide anions
ONOO ⁻	Peroxynitrite
PARP	Poly (ADP-ribose) polymerase
PD	Parkinson's disease
ROS	Reactive oxygen species
SMA	Supplementary motor cortex
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SOD	Superoxide dismutase
STN	Subthalamic nucleus
STN-HFS	High-frequency stimulation of the subthalamic nucleus
TH	Tyrosine hydroxylase

Introduction

The correct execution of voluntary movements is controlled by the so-called *motor circuit* of the brain. Voluntary movements appear to be initiated at the cortical level where there is activation of excitatory glutamatergic and inhibitory GABA-ergic projections to the basal ganglia (Wichmann and DeLong 2006) that process these signals producing an output signal that returns to the cortex, through the thalamus, to modulate movement execution (Blandini et al. 2000; Wichmann and DeLong 2006). The basal ganglia are a group of related subcortical nuclei, including the neostriatum (caudate nucleus and putamen), the ventral striatum, the external and internal segments of the globus pallidus (GPe and GPi respectively), the subthalamic nucleus (STN), and the substantia nigra pars reticulata and compacta (SNr and SNc, respectively). These structures are components of circuits involving portions of the cerebral cortex, thalamus and brainstem (Blandini et al. 2000; Wichmann and DeLong 2006).

Parkinson's disease (PD) is the most common basal ganglia disorder in which the ability to control voluntary movements is lost as a consequence of profound changes in the functional organization of the basal ganglia nuclei (Ehringer and Hornykiewicz 1960). PD manifests itself in varying combination of symptoms such as tremor at rest, rigidity, bradykinesia, and loss of postural reflexes. The neuropathological hallmark of PD is the selective degeneration of dopaminergic (DA-ergic) neurons in the nigrostriatal system (Jellinger 1989; Scherman et al. 1989). The pathogenesis of PD is multifactorial, genetic, and environmental events. Important factors include: formation of free radicals, impaired mitochondrial activity, increased sensitivity to apoptosis, excitotoxicity, and inflammation (Esposito et al. 2002,

2007a, 2007b; von Bohlen und Halbach et al. 2004; Di Giovanni 2007, 2008). Nevertheless, the progressive nature of PD and the observation that neuronal degeneration in the SNc is slow and protracted (Fearnley and Lees 1991) offers good opportunities for therapeutic intervention. It appears clear that understanding the etiopathogenesis of PD, the modalities whereby the neurodegenerative process begins and progresses, is fundamental to the development of drugs to slow or prevent the progression of PD. There have certainly been major advances in these areas over the past few years, but the modalities whereby the neurodegenerative process begins and progresses remain unclear. Most of the progress in this field has been gained thanks to the toxin-models of PD. Although they have undoubtedly been useful, it is worth emphasizing that preclinical research on animal models of PD has sometimes provided discrepant results from the evidence in humans, highlighting that these experimental models represent an imperfect replica of human disorders (Scholtissen et al. 2006; Waldmeier et al. 2006). Here we review the most prominent data obtained from studies that have utilized the intracerebral microdialysis technique coupled to in vivo animal models of PD.

Pathophysiology of Parkinson's Disease

The striatum (or caudate-putamen) is the main input nucleus of the basal ganglia, which receives topographical excitatory projections from almost the entire cerebral cortex, especially from the sensorimotor and frontal cortex (Parent 1996). The striatum and the downstream structures in the basal ganglia are organized in topographically and functionally segregated pathways (Fig. 1). The cortical inputs to the striatum are convergent in such a way, for example, that sensory and motor cortex areas converge into single striatal zones (Flaherty and Graybiel 1991). Close to the striatum is located the GPi and the SNr, the main output nuclei of the basal ganglia (DeLong 1990). They project, via various thalamic nuclei, to most cortical areas of the frontal lobe (Alexander et al. 1990). This architecture means that the basal ganglia are part of extensive loops, basal ganglia-thalamocortical circuits, which link almost the entire cerebral cortex to the frontal lobe. The striatum can be divided into three main parts: the putamen, the caudate nucleus, and the ventral striatum, as already mentioned. This division roughly corresponds to a functional division of basal ganglia-thalamocortical circuits: (sensori)motor circuits of the putamen, with output to primary motor cortex, the supplementary motor cortex (SMA), and the premotor cortex; associative circuits of the caudate nucleus, with output to the prefrontal cortex; and limbic circuits of the ventral striatum, with output to the anterior cingulate cortex and medial prefrontal cortex

(DeLong 1990; Parent 1996). The striatum projects to the output structures (GPi and SNr) by two pathways, the so-called *direct* and *indirect* pathways. The *indirect* pathway also includes the STN. All the projections from the striatum, the GPe, the GPi, and SNr release GABA and are inhibitory, while the projections from the cortex, the STN, and the thalamus are excitatory, and use glutamate (GLU) as their neurotransmitter (Blandini et al. 2000; Wichmann and DeLong 2006). The GABA-containing neurons in the GPi and the SNr are tonically active; they project to the ventral tier of thalamus (ventrolateral, ventromedial, ventral anterior nuclei) and form inhibitory synaptic contacts with thalamo-cortical neurons that project to the motor and premotor cortex (Fig. 1). The activation of the *direct* pathway inhibits GPi/SNr neurons, which in turn disinhibits thalamic neurons, finally resulting in the excitation of the cortical neurons. The

activation of the *indirect* pathway has an opposite effect, activating the GPi/SNr and thereby inhibiting the cortex (DeLong 1990; Blandini et al. 2000; Wichmann and DeLong 2006). In this way, the two pathways balance each other, modulating cortical activity.

Among the basal ganglia nuclei, the striatum seems to have a prominent role in determining when a given motor program should be selected and called into action. For the hypothesized function of the striatum in selection of motor programs, a certain level of tonic DA activity is required. Dopamine projections from the SNc to the striatum modulate the activity of striatal neurons in a complex way. According to a simplified model, the striatal neurons forming the *direct* pathway mainly express excitatory D₁-receptors, while the striatal neurons in the *indirect* pathway mainly have inhibitory D₂-receptors. This means that DA would facilitate

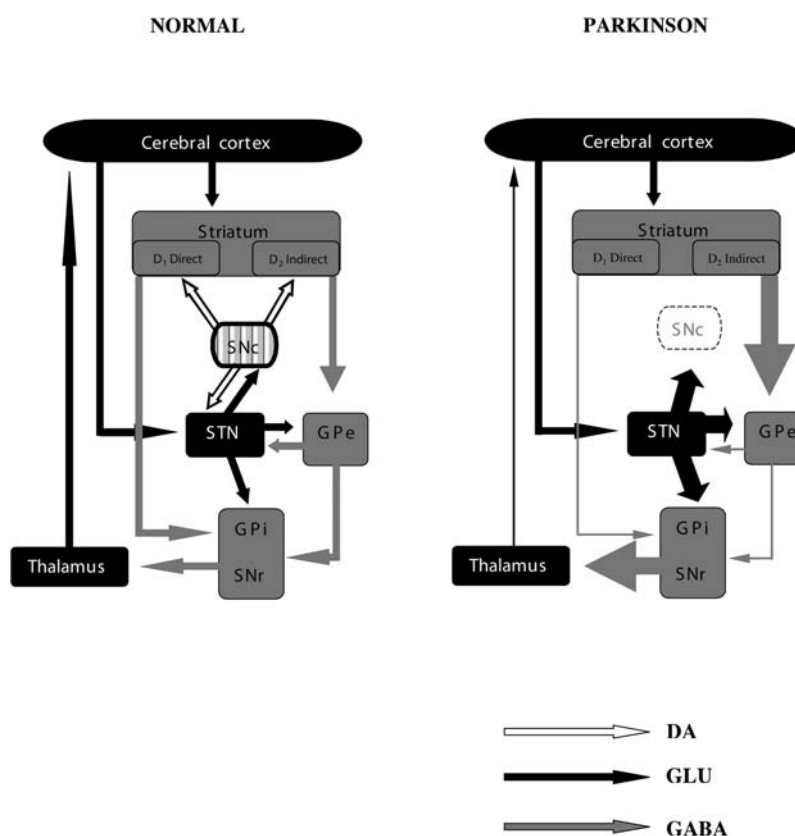


Fig. 1 Simplified diagram (modified from Blandini et al. 2000) illustrating the changes occurring in the basal ganglia functional organization in Parkinson's disease, with respect to normal condition. Relative thickness of arrows indicate the degrees of activation of the transmitter pathways. The basal ganglia participate in larger circuits that also include cortex and thalamus. The striatum is the principal input structure of the basal ganglia, and the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr) are the major output structures, projecting toward the thalamus and brainstem. According to conventional anatomical models, basal ganglia input and output structures are linked via a monosynaptic "direct" pathway and a polysynaptic "indirect" pathway that involves the external pallidal segment (GPe) and the subthalamic nucleus (STN). Dopamine released from terminals of the nigrostriatal (SNc) projection is thought to modulate basal ganglia activity by inhibiting activity along the "indirect" pathway through stimulation of dopamine D₂ receptors and enhancing activity along the "direct" pathway by the stimulation of the dopamine D₁ receptor. The same model has been applied to explain aspects of the pathophysiology of parkinsonism. Loss of striatal dopamine is believed to result in increased striatal inhibition of GPe, leading to disinhibition of STN neurons and to increased basal ganglia output from GPi and SNr. Increased and altered basal ganglia output to the thalamus is thought to disturb cortical processing, which is ultimately responsible for the development of many of the parkinsonian motor signs. Dopamine (DA) unfilled arrows, glutamate (GLU) black arrows, γ-aminobutyric acid (GABA) grey arrows

motor behaviors through the activation of the *direct* pathway and conversely through the inhibition of the *indirect* one (O'Connor 1998; Blandini et al. 2000; Wichmann and DeLong 2006). Reduced DA innervation of the striatum results, indeed, in hypokinesia and difficulty in initiating different motor patterns, including facial expression (Blair 2003). Dopamine depletion in PD causes a series of changes in the basal ganglia that may not begin with alterations in the striatopallidal circuits but rather with changes at the level of the subthalamic motor nuclei (i.e., glutamatergic) (Fig. 1). The capacity of tonic DA release to modulate basal ganglia circuitry permits compensation for the effects of nigral DA cell degeneration in the early stages of parkinsonism (Obeso et al. 2004). In the early stages, the excess stimulation of GPi/SNr resulting from overactivity in the STN may be compensated for by an increased inhibitory input from the GPe (Obeso et al. 2004). The functional hallmark of the Parkinsonian state is an increased neuronal activity in the output nuclei of the basal ganglia, leading to an excess inhibition of the thalamocortical and brainstem motor systems (Albin et al. 1989; DeLong 1990) (Fig. 1). According to the classic pathophysiologic model of the basal ganglia, DA deficiency leads to the loss of the D₁-receptor-mediated stimulation of the *direct* pathway that becomes underactive, while the *indirect* pathway becomes overactive due to the loss of a D₂-receptor-mediated inhibition with a consequently reduced inhibition of GABA-ergic striatal neurons in the *indirect* pathway and a decreased facilitation of GABA-ergic

neurons in the *direct* pathway (Albin et al. 1989; DeLong 1990). The reduced inhibition of neurons in the *indirect* pathway would lead sequentially to an overinhibition of the GPe, disinhibition of the STN, and excessive glutamatergic drive of the GPi and SNr. Similarly, the decreased activation of neurons in the *direct* pathway reduces its inhibitory influence on GPi/SNr and contributes to the excessive basal ganglia output activity (Fig. 1). With continued neurodegeneration, the striatal DA deficit increases, leading to an excess inhibition of the GPe. In turn, hypoactivity of the GPe reduces its inhibitory output onto the GPi and STN, which now become more overtly hyperactive with a consequent aggravation of the Parkinsonian motor states (see Blandini et al. 2000; Obeso et al. 2004; Wichmann and DeLong 2006 for reviews). These changes are thought to represent the neural substrate for Parkinsonian motor symptoms. Therefore, strategies to restore the balance of these pathways might be beneficial to treating PD.

Microdialysis to Study Parkinson's Disease

Microdialysis coupled to high-performance liquid chromatography (HPLC) is an established technique for studying physiological, pharmacological, and pathological changes of a wide range of low molecular weight substances in the brain extracellular fluid. It is based on the evidence that a

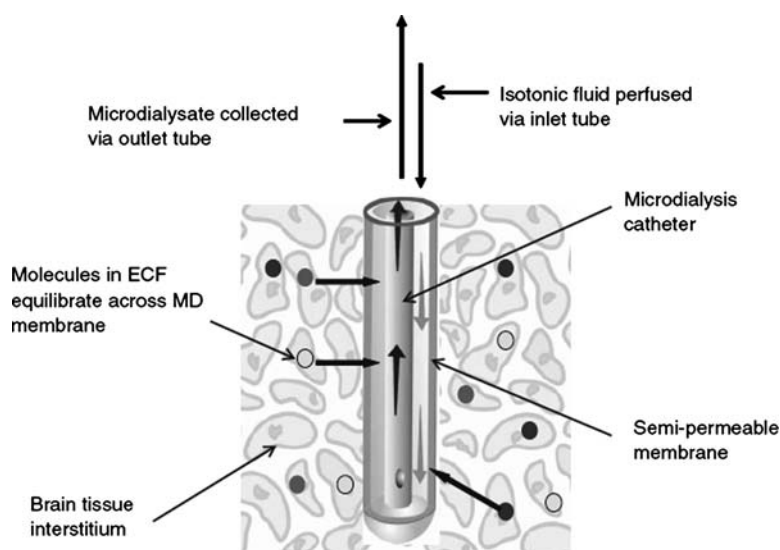


Fig. 2 *The Microdialysis Probe*: A microdialysis (MD) probe is usually constructed as a concentric tube where the perfusion fluid enters through an inner tube; flows to its distal end; exits the tube and enters the space between the inner tube and the outer dialysis membrane. The direction of flow is now reversed and the fluid moves toward the proximal end of the probe. This is where the “dialysis” takes place, i.e. the diffusion of molecules between the extracellular fluid (ECF) and the perfusion fluid. Vice versa, by reverse-dialysis is possible the introduction of a substance into the extracellular space via the microdialysis probe. The inclusion of a higher amount of a drug in the perfusate allows the drug to diffuse through the microdialysis membrane to the tissue. This technique not only allows the local administration of a substance but also permits the simultaneous sampling of the extracellular levels of endogenous compounds. From Tisdall and Smith (2006), with permission from Oxford University Press

probe (Fig. 2) made of a hollow fiber permeable to solutes of low molecular weight inserted into the brain tissue mimics blood capillaries in exchanging material from and to the extracellular fluid (Benveniste 1989; Ungerstedt 1991; Tisdall and Smith 2006). Microdialysis has been employed during the last 25 years by several authors primarily to study brain function and changes in the levels of endogenous compounds such as neurotransmitters or metabolites (Höcht et al. 2007). Nevertheless, in central nervous studies, reverse microdialysis has been extensively used for the study of diverse pharmacological and toxicological agents, such as antidepressants, antipsychotics, antiparkinsonians, hallucinogens, drugs of abuse, and experimental drugs on the local effects on neurotransmission at different central nuclei. Thus, the microdialysis approach has largely contributed not only to the clarification of the physiological role of the various neuronal systems but also to the development of therapeutic strategies for the treatment of a number of neuropsychiatric disorders.

Microdialysis and Oxidative Stress

Oxidative stress is defined as an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates and/or easily repair the resulting damage. Increasing evidence suggests that reactive oxygen species are involved in the pathophysiology of PD. The microdialysis technique is suitable for measuring radical formation in the extracellular space in vivo. Indeed, after probe implantation and obtaining a stable baseline, the probe can be used to deliver simultaneously the stimulating agent (e.g. 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenylpyridinium ion (MPP⁺) and the trapping agent (see below) for the radical determination,

without any purification steps before HPLC analysis. In addition, the implanted microdialysis probe allows reliable recording for many hours, and the animal serves as its own control. Due to the high reactivity and short half-life of ROS in biological tissue, indirect methods for in vivo detection have been widely used. The method for in vivo measurement of the hydroxyl radicals ($\cdot\text{OH}$) is based on their ability to attack the benzene rings of aromatic molecules (Fig. 3). The salicylate (2-HBA) trapping technique is the most widely used technique for ROS detection in vivo and has previously been employed in experimental animal models of PD with microdialysis and HPLC coupled with an electrochemical detector (ECD) (Chiueh et al. 1992a,b; Obata and Chiueh 1992; Obata et al. 2001a; Teismann et al. 2001; Obata 2006a). However, when ROS react with salicylate two adducts: 2,3- and 2,5-DHBA are formed (Fig. 3). Concerns have been raised with regard to the specificity of the salicylate trapping technique, since enzymatic formation of 2,5-DHBA occurs at extracerebral sites by the cytochrome *p* 450 system in the liver after systemic administration. In addition, 2,3-DHBA is metabolized in the rat intestine, making it an unspecific marker (Halliwell et al. 1991; Montgomery et al. 1995; Halliwell and Kaur 1997). Salicylate may also chelate divalent metal ions (Ste-Marie et al. 1996), thus affecting hydroxyl radical formation. Finally, salicylate has been shown to affect the inducible nitric oxide synthase (iNOS) (Amin et al. 1995). Therefore, when a trapping method for reactive oxygen species (ROS) detection is employed, a very careful evaluation of possible sources of spontaneous ROS formation may be made and these sources may be eliminated as far as possible. Plausible factors affecting the hydroxylation of DHBAs using microdialysis technique include time, use of metal ions, and reuse of microdialysis probes. The liquid switch perfusion with an artificial physiological solution may possibly corrode some metal surfaces. Reusing

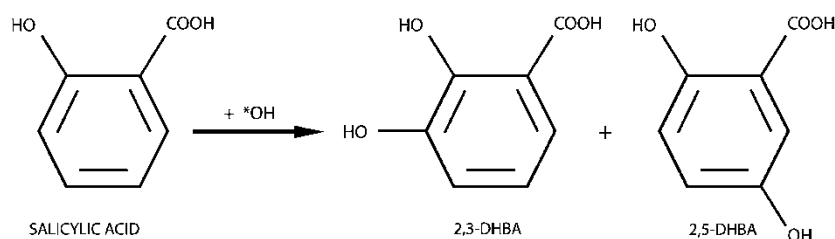


Fig. 3 Reaction scheme of salicylate (2-HBA) with the hydroxyl radicals ($\cdot\text{OH}$) to form 2,3- and 2,5-dihydroxybenzoate (2,3- and 2,5-DHBA) and 4-hydroxybenzoic acid (4-HBA) hydroxylation that forms the 3,4-dihydroxybenzoic acid (3,4-DHBA)

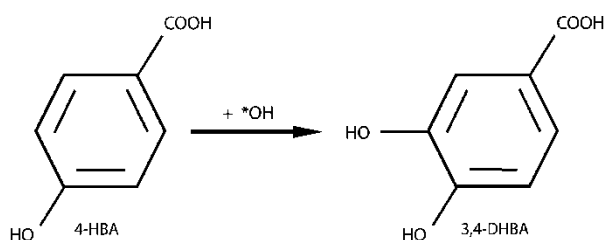
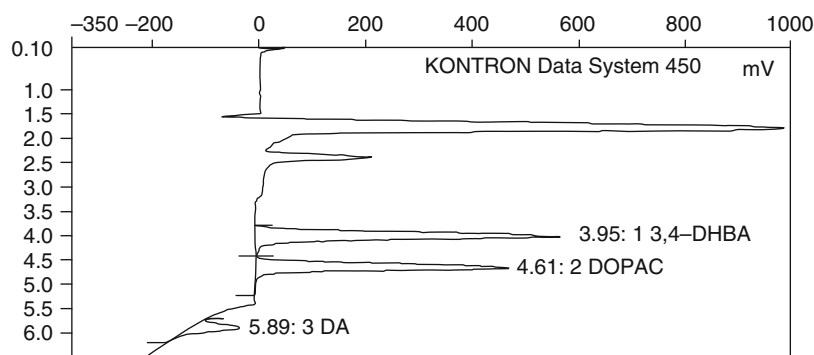


Fig. 4 Chromatogram of 3,4-dihydroxybenzoic acid (3,4-DHBA), dihydroxyphenyl acetic acid (DOPAC) and dopamine (DA)



the microdialysis probe resulted in an increased DHBA production (Montgomery et al. 1995). Therefore, to reduce artifactual exogenous hydroxyl free radical production, all stainless steel may be eliminated from the microdialysis setup and HPLC system, and the perfusing solutions may kept cold, shielded from the light and freshly prepared just before use. A sensitive and specific hydroxybenzoate (4-hydroxybenzoic acid; 4-HBA) hydroxylation assay is also used in conjunction with HPLC-ECD to monitor $\cdot\text{OH}$ production (Ste-Marie et al. 1996; Liu et al. 2002). 4-HBA is known to trap $\cdot\text{OH}$ with equal efficacy as salicylate (Ste-Marie et al. 1996). It offers a number of advantages compared with the use of salicylate: first, only one isomer is formed by the hydroxylation of 4-HBA, the 3,4-DHBA (Fig. 4), and therefore the signal due to $\cdot\text{OH}$ attack is not split between two compounds (2,3- and 2,5-DHBA), decreasing the sensitivity of the assay, as in the case of salicylate (Fig. 3). Second, the systemic production of 3,4-DHBA following the administration of a large bolus of 4-HBA in vivo is negligible compared with the in vivo enzymatic hydroxylation of salicylate. Last, salicylate is an effective chelator of divalent metal ions, including Fe^{2+} . As Fe^{2+} catalyzes the decomposition of H_2O_2 to $\cdot\text{OH}$, this may result in an overestimation of apparent $\cdot\text{OH}$ production due to its increased local formation (Ste-Marie et al. 1996; Liu et al. 2002). On the other hand, the formation of 3,4-DHBA may reflect the production of either $\cdot\text{OH}$ or other highly reactive species such as peroxynitrite produced by the reaction between superoxide anions and nitric oxide (Bogdanov et al. 1999). Thus, the formation of 3,4-DHBA likely reflect ROS production but is not specific to any single radical species.

6-OHDA Model: Microdialysis studies

6-OHDA is a hydroxylated analog of the natural neurotransmitter DA that induces specific damage via oxidative stress, by using the catecholamine transport system of DA and norepinephrine (Lotharius et al. 1999; see Glinka et al.

1997; Blum et al. 2001; Betarbet et al. 2002 for review). 6-OHDA is unable to cross the blood–brain barrier and must be administered intracerebrally to exert its toxic effects (see Beal 2001; Blum et al. 2001; Betarbet et al. 2002 for review). It has been reported that 6-OHDA-induced neuron degeneration involves the processing of hydrogen peroxidase and $\cdot\text{OH}$ in the presence of iron (Sachs and Jonsson 1975). Furthermore, it has been shown that 6-OHDA treatment reduces striatal glutathione (GSH) and superoxide dismutase (SOD) enzyme activity, and seems to be toxic to mitochondrial complex I leading to the formation of superoxide free radicals (see Glinka et al. 1997; Blum et al. 2001; Betarbet et al. 2002; Schober 2004; Simola et al. 2007 for review). Taken together, in neurodegenerative processes, 6-OHDA causes respiratory inhibition and oxidative stress, induced by free radical formation (see Beal 2001; Betarbet et al. 2002; Emborg 2004; Schober 2004 for review). This experimental Parkinsonian model has been produced in many species, including mice, rats, cats, dogs, and nonhuman primates. Nevertheless, the rat is the most commonly used species due to the replicability and the stability of the induced model. Typically, the lesions are unilateral and injections are made into the medial forebrain bundle (Perese et al. 1989), the SN (Ungerstedt 1968) or into the striatum (Sauer and Oertel 1994). Depending on the location of the injection site, the number of injection sites, and the amount of neurotoxin, the animals present different time-courses of progression and severity of the lesion (see Betarbet et al. 2002; Deumens et al. 2002; Schober 2004 for review). Middle forebrain bundle or nigral targets induce an almost-complete nigral lesion (> 90% nigral cell loss) within 1–3 days, which is equivalent to end stage in PD (Beal 2001; Betarbet et al. 2002; Emborg 2004; Schober 2004). The other 6-OHDA model involves injection of the toxin directly into the striatum to cause retrograde degeneration of SNc neurons (Sauer and Oertel 1994; Przedborski et al. 1995). This causes slow partial lesion of these neurons (>70% nigral cell loss) over four weeks and has been used to mimic the slow progression of PD (Beal 2001; Betarbet et al. 2002; Emborg 2004; Schober 2004). Several authors have shown that in the

striatum of 6-OHDA-lesioned rats dialysate DA and its metabolite dihydroxyphenyl acetic acid (DOPAC) levels decrease only moderately, despite an 80–95% loss of DA-ergic neurons (Robinson and Whishaw 1988; Castañeda et al. 1990; Wachtel and Abercrombie 1994; Sarre and Michotte 1996; Miller and Abercrombie 1999; Jonkers et al. 2000). These authors suggested that presynaptic compensatory changes in the remaining DA-ergic neurons exist to normalize extracellular DA concentrations and this contributes to the recovery of function. When the loss of DA-ergic cells exceeds the limit of 95%, the compensatory mechanisms are no longer sufficient and DA levels decrease dramatically. Although Hoffman et al. (1997) observed reduced dialysate DA and DOPAC levels in the SN of 6-OHDA-lesioned rats, other authors reported that basal dialysate DA and DOPAC levels in the SN were similar to those observed in normal rats (Jonkers et al. 2000; Bergquist et al. 2003; Sarre et al. 2004), as seen in the striata of lesioned rats. Compensatory mechanisms also exist in the SN to maintain extracellular DA and DOPAC after DA-ergic denervation. Indeed, the remaining DA-ergic neurons in the SN increase their rate of discharge, and electrophysiological data suggest, in contrast to the striatum, that diffusion, rather than uptake, is the most important determinant of the DA time course in the SN (Cragg et al. 2001). Two different pools contribute to basal DA levels in the SN: a fast sodium channel-dependent portion and a TTX-insensitive one originating from diffusion of DA (Sarre et al. 2004), supporting the idea that volume transmission might contribute to the nigral DA levels. In addition, reduced DA reuptake and catabolism with a simultaneous increase of DA synthesis in the SN (Sarre et al. 2004) as in the striatum (Miller and Abercrombie 1999) contributes to maintain extracellular DA and DOPAC after DA-ergic denervation. Interestingly, amphetamine-induced DA release in both the SN (Hoffman et al. 1997; Sarre et al. 2004) and the striatum (Robinson and Whishaw 1988; Castañeda et al. 1990) is attenuated by the lesion, compared with the intact side. As long as the lesion size did not exceed 95%, the amphetamine-induced DA release in DA-depleted striatum was comparable to that in intact striatum, when the DA depletion was >95%, dialysate DA levels no longer increased after the systemic administration of amphetamine (Robinson and Whishaw 1988; Castañeda et al. 1990), this meaning that at least 5% of nigral cells are required to maintain a relatively normal extracellular concentration of DA necessary for any significant recovery of function.

The loss of DA-ergic neurons in the SNc after 6-OHDA lesions (and in PD) is associated with an imbalance in the activity of the other structures of the basal ganglia circuitry that is reflected in changes in the release of the others neurotransmitters, particularly GLU and GABA.

Striatal GLU release appears to be under the inhibitory control of DA (Yamamoto and Davy 1992; Robinson et al.

2003), and therefore, variable and time-dependent effects on basal GLU levels following 6-OHDA in the striatum have been found. Increased basal levels of striatal GLU in lesioned rats were observed by several authors (Tossmann et al. 1986; Lindefors and Ungerstedt 1990; Yang et al. 2006a). Moreover, effects of DA denervation on extracellular striatal GLU levels were reported to undergo biphasic changes, namely an increase after 3 weeks and a decrease 11 weeks post lesioning (Meshul et al. 1999; Jonkers et al. 2002; Touchon et al. 2005). On the contrary, a decrease of GLU after a month and its increase three months later was reported in the SNr (Touchon et al. 2005). These time-dependent changes of GLU overflow were explained as a consequence of an initial compensatory response following the first month after the lesion that induced the activation of the thalamo-cortico-striatal pathway, leading to a decrease in the output of this pathway after three months (Touchon et al. 2005). However, some groups reported unchanged extracellular GLU levels after 6-OHDA in the striatum and SNr (Abarca and Bustos 1999; Marti et al. 2002; Galeffi et al. 2003; Robelet et al. 2004). Others showed that GLU levels increased 2 weeks post lesioning in the SNr and entopeduncular nucleus (You et al. 1996), functionally similar to the SNr, in rats (Biggs et al. 1997). Striatal GABA concentrations were unaffected (Bianchi et al. 2003) or enhanced (Tossmann et al. 1986; Lindefors et al. 1989; Lindefors and Ungerstedt 1990; Abarca and Bustos 1999; Ochi et al. 2000) after DA-ergic denervation. No changes occurred in extracellular levels of both GLU and GABA in the STN (Ampe et al. 2007). SNc lesions induced a dramatic increase in the extracellular GLU level in both SNr and GP (Lindefors et al. 1989; Ochi et al. 2004a; Windels et al. 2005). Such observations thus provide the neurochemical confirmation of the hyperactivity of subthalamopallidal and subthalamonigral pathways induced by DA depletion. An increase in GABA levels was also measured in the GP of hemiparkinsonian rats (Ochi et al. 2000; Bianchi et al. 2003; Windels et al. 2005). On the other hand, no change of basal GABA concentrations in the SNr was shown, and this result may be attributable to the complex GABA-ergic systems in the SNr arising from its anatomical features (Bianchi et al. 2003; Ochi et al. 2004a; Windels et al. 2005).

Oxidative stress may contribute to nigral cell death in PD. Several investigations show increased iron levels, decreased levels of reduced glutathione (GSH), and impaired mitochondrial function (Sayre et al. 2008 for review). This leads to the oxidative damage, because lipid peroxidation is increased in SN and there is a widespread increase in protein and DNA oxidation in the brain in PD (see Jenner 2003 for review). As already mentioned, when perfused into the striatum by reverse microdialysis, 6-OHDA is accumulated by DA-ergic terminals and then retrogradely transported in the cell bodies of DA-ergic neurons, where it is oxidized (Glinka et al. 1997;

Blum et al. 2001), inducing a massive release of hydroxyl free radicals (Opacka-Juffry et al. 1998; Ferger et al. 2001a; Themann et al. 2001) and peroxynitrite (Ferber et al. 2001b). The observed $\cdot\text{OH}$ production is associated with a parallel, massive increase in extracellular striatal DA (Opacka-Juffry et al. 1998; Ferger et al. 2000) likely to effect an insult to synaptic function (Opacka-Juffry et al. 1998). On the other hand, this massive overflow of DA is likely to be the earliest response of DA neurons to 6-OHDA, and endogenous DA may exacerbate 6-OHDA toxicity either by aggravating oxidative stress or through direct interactions with cellular regulatory processes (Opacka-Juffry et al. 1998). Furthermore, the oxidation of DA by enzymatic and nonenzymatic mechanisms produces neuromelanin, which potentiates hydroxyl radical formation when combined with iron (Jenner et al. 1992). In this early stage, 6-OHDA produces enhanced levels of oxidative DNA base modifications, leading to the damage of DNA (Ferber et al. 2000), and also acts as a potent inhibitor of the mitochondrial respiratory complexes I and IV (Glinka et al. 1997), which then starts a sequence of toxic processes resulting in cell death.

MPTP/MPP⁺ Models: Microdialysis studies

The toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a byproduct of illegally manufactured synthetic meperidine derivative, caused a syndrome that strikingly resembled PD, both at clinical and pathological levels, in drug addicts (Langston et al. 1983; Hornykiewicz 1989). It is a potent neurotoxin that produces selective destruction of nigrostriatal DA-ergic neurons in humans and animals and therefore elicits neurochemical and neuropathological changes that are similar to idiopathic PD. It seems, however, that the mouse MPTP model provides the most useful animal model of PD to study neuropathological and neurochemical changes (see Schmidt and Ferger 2001; Betarbet et al. 2002 for review). In contrast, rats are relatively insensitive to MPTP (Giovanni et al. 1994a), since these, injected with doses of MPTP or MPP⁺ comparable to those in mice, exhibited at least an order of magnitude less DA-ergic neurodegeneration (Giovanni et al. 1994a, 1994b) in part due to a more pronounced vesicular sequestration of MPP⁺ in rats (Staal and Sonsalla 2000). MPTP itself is highly lipophilic and readily crosses the blood–brain barrier. Its toxicity is induced through conversion to MPP⁺ in astrocytes by monoamine oxidase B (Chiba et al. 1984), which is then actively accumulated into DA-ergic neurons (Rollema et al. 1988a; Santiago et al. 1995; Gainetdinov et al. 1997). Its specific selectivity seems to be closely related to the DA uptake system (DAT), indeed, the intrastriatal infusion of MPP⁺ caused an immediate and massive 300-fold release of DA

in DAT+/+ mice, whereas the same concentration produced only a six-fold increase of DA output in the striatum of DAT−/− mice, demonstrating that the accessibility of the neurotoxin to DA-ergic cells via the DAT represents an absolute requirement for MPTP toxicity (Gainetdinov et al. 1997). Once inside the neuron, MPP⁺ accumulates within mitochondria where it acts by inhibiting the electron transport system of the mitochondrial complex I (NADPH-ubiquinone oxidoreductase I), leading to an impairment in ATP production (Fabre et al. 1999), an elevation of the intracellular calcium concentration and to the generation of free radicals (Sun et al. 1988; Obata et al. 2002a), resulting in cellular energy failure (Nicklas et al. 1985, 1987), and the formation of superoxide anions (O_2^-) (Dawson 2000). These, coupled with generation of both neuronal- and microglial-derived NO, form peroxynitrite (ONOO^-), which oxidatively injures DA neurons, perhaps through DNA damage, and the activation of the highly energy-consuming DNA repair enzyme poly (ADP-ribose) polymerase (PARP) promotes further ATP depletion, thus contributing to DA neuron death (see Grünewald and Beal 1999; Obata 2006a for review).

The major part of MPP⁺ toxicity may be attributed to the increase in oxidative stress, as studied by microdialysis (Chiueh et al. 1992a,b; 1994; see Obata 2002 for review), in which free iron is involved. The damage is produced when these $\cdot\text{OH}$ react with transition metals, such as iron with the creation of even more reactive oxygen species (Chiueh et al. 1992a,b, 1994; Matarredona et al. 1997; Santiago et al. 1997, 2000; Obata 2006b). Furthermore, acute MPP⁺ induced destruction of DA-ergic nerve terminals or cell bodies is also associated with a massive increase in the extracellular content of DA (Rollema et al. 1986, 1988a,b; Santiago et al. 1991a,b, 1997; Obata et al. 2001a) that itself produces toxic effects through its metabolism and auto-oxidation, leading to the production of hydrogen peroxide, which if not reduced by cellular mechanisms, can react with transition metal, such as iron, to form further hydroxyl radicals, thus priming a vicious circle with the consequent cell damage (Obata and Chiueh 1992; Chiueh et al. 1993; Matarredona et al. 1997; Santiago et al. 2000; Obata 1999a, 2002, 2006b; Obata et al. 2001a; Obata and Kubota 2001). In this respect, it is interesting to note that systemic MPTP caused a significant depletion of glutathione (GSH) in both the SNc and the striatum of mice (Mohanakumar et al. 2000). On the other hand, during the perfusion of MPP⁺ into the rat striatum or SNc, GSH maintained normal basal concentrations in quantities sufficient to scavenge most $\cdot\text{OH}$ (Han et al. 1999). The fact that MPTP/MPP⁺ causes the loss of nigrostriatal GSH without corresponding increases of glutathione disulfide (GSSG) (Foster et al. 2003) suggests that factors other than excessive ROS production are also able to induce MPTP/MPP⁺ parkinsonism. Intrastriatal infusion of MPP⁺, however, causes also a massive release of GLU and other excitatory

aminoacids (Carboni et al. 1990; Yang et al. 1995, 2006b; Ferger et al. 1998). Consequently, GLU may exert its neurotoxic properties in a dual way: direct excitotoxicity caused by its excessive release with a consequent activation of GLU receptors (in particular of the N-methyl-D-aspartate (NMDA) type) that leads to an intracellular accumulation of Ca^{++} , which in turn, initiates a cascade of alterations resulting in the formation of ROS (Yang et al. 1995; Ferger et al. 1998) as well as to a reduced intracellular glutathione synthesis (Schmidt and Ferger 2001), and indirect excitotoxicity based on impaired mitochondrial function and a cascade of events which enable normally “non-toxic levels” or lower levels of GLU to become cytotoxic (Beal et al. 1993; Schmidt and Ferger 2001). Because of this, the biochemical changes due to MPTP/MPP⁺ are reflected in marked depletion of DA and its free acid metabolites, homovanillic acid (HVA) and DOPAC in the nigrostriatal system (Skirboll et al. 1990; Santiago et al. 1991a,b; Robinson et al. 2003).

As already discussed, DA released from terminals of the nigrostriatal projection is thought to modulate basal ganglia activity by inhibiting activity along the *indirect* pathway and enhancing activity along the *direct* pathway (Albin et al. 1989; Blandini et al. 2000; Wichmann and DeLong 2006). Given the polarity of connections along *direct* and *indirect* pathways, striatal release of DA may result in an overall reduction of basal ganglia output. The same model has been applied to explain aspects of the pathophysiology of parkinsonism. The loss of striatal DA is believed to result in increased striatal inhibition of GPe, leading to the disinhibition of STN neurons and to increased basal ganglia output from GPi and SNr (Fig. 1) (Albin et al. 1989; DeLong 1990). Increased and altered basal ganglia output to the thalamus is thought to disturb cortical processing, which is ultimately responsible for the development of many of the Parkinsonian motor signs (see Blandini et al. 2000; Obeso et al. 2004; Wichmann and DeLong 2006 for review). In agreement, following an extensive nigrostriatal lesion by MPTP there was an increased release of GABA in the GPe (Robertson et al. 1991; Schroeder and Schneider 2002) that in turn reduced the release of GABA in the STN (Soares et al. 2004) resulting in the disinhibition of STN and increased basal ganglia output.

An Intriguing Model: Two Day Microdialysis Method

The 2-day test-challenge microdialysis method for the study of PD was developed by Rollema and colleagues in the late 1980s (Rollema et al. 1986, 1988b, 1990). The technique has been used extensively to characterize the neurotoxicity of a variety of compounds in the striata of mice, monkeys, and

rats (Rollema et al. 1989, 1990, 1994; Booth et al. 1989; Giovanni et al. 1994b; Santiago et al. 2000, 2001; Staal and Sonsalla 2000; Foster et al. 2003).

This rapid sampling procedure is made in two days: in the first day of the experiment a fixed toxin (e. g. MPP⁺ or 6-OHDA) dissolved in Ringer solution is perfused through the microdialysis probe, in a target area of the striatum or SNc for a short time (10–15 min) with a fixed concentration (generally 1–10 mM, or more, depending from the used substance), to induce neurodegeneration of the nigrostriatal system. Forty-eight hours later (day 2), the amount of DA released by the perfusion (challenge) of a second dose of MPP⁺ could be indicative of the damage produced by a previous perfusion of a toxic compound, since it could be proportional to the number of remaining DA-ergic terminals (Fig. 5). Indeed, the massive DA extracellular output after the first MPP⁺ perfusion is an index of DA-ergic cell disruption as has been previously hypothesized by Rollema et al. (1988b), who showed a marked increase of DA and lactate release on the first day, indicating the inhibition of mitochondrial respiration and a subsequent inhibition of the electron flow, that induces a dramatic reduction of DA release after the challenge with the second perfusion of the toxin, proportional to the surviving DA-ergic cells. Furthermore, Giovanni et al. (1994b) demonstrated that the decreases in DA overflow observed on day 2, after the challenge dose, reflect DA nerve terminal degeneration as determined by reductions in striatal tissue concentrations of DA when measured 1 week after infusion of MPP⁺ into the striatum. The effects of intranigral MPP⁺ perfusion were also studied (Rollema et al. 1994; Matarredona et al. 1997), and subsequently 6-OHDA was also used to induce striatal degeneration employing this procedure (Santiago et al. 2001; Di Matteo et al. 2006a) (Fig. 5). Thus, this method is suitable to study different drug-induced DA-ergic toxicity in the nigrostriatal system and also to estimate the quantitative damage induced by these toxins. Numerous neuroprotective experimental studies have been conducted using this method, demonstrating the ability of the iron chelator desferrioxamine to protect the SN (Matarredona et al. 1997) and the striatum (Santiago et al. 1997) against MPP⁺ toxicity. In addition, the activation of group II metabotropic GLU (mGlu) receptors (Matarredona et al. 2001), the inhibition of NMDA receptors by MK-801 (Santiago et al. 1992), and the inhibition of the NOS by pretreatment with 7-nitroindazole (7-NI) showed a protective effect against neuronal damage induced by intra-striatal infusion of MPP⁺ (Di Matteo et al. 2006b). Finally, aspirin has a protective effect against MPP⁺ and 6-OHDA-induced neurodegeneration (Di Matteo et al. 2006a). Interestingly, this method can be employed also to study neuroregenerative strategies, since Santiago and colleagues (1991a,b) investigated whether a short-lasting infusion of MPP⁺ into the SN

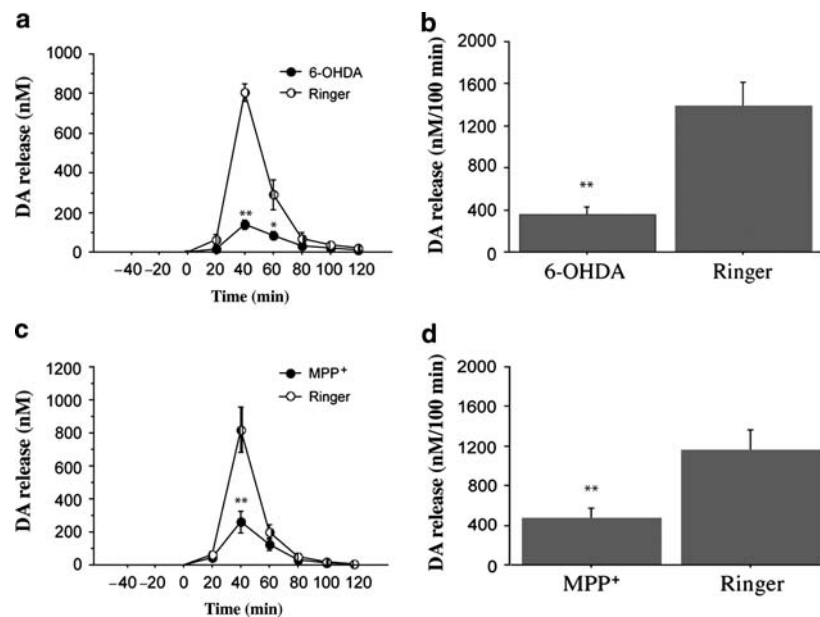


Fig. 5 The 2-day test-challenge microdialysis method. Time course of the effect of 15 min 1 mM MPP⁺ perfusion 24 h later after perfusion of 6-OHDA (1 mM for 15 min) (panel A), and 1 mM-15 min MPP⁺ (panel C) on extracellular dopamine output in the corpus striatum. Each data point represents mean $\pm$ SEM of absolute levels of DA, without considering probe recovery. Statistical analysis shows that MPP⁺ and 6-OHDA perfused in the first day of the experiment induced neurodegeneration of nigrostriatal pathway as shown by the decrease in DA release after MPP⁺ challenge in the second day (one-way ANOVA, followed by Fisher's PLSD *post hoc* test: * $p < 0.05$; ** $p < 0.01$ 6-OHDA vs. Ringer (Control group), and ** $p < 0.01$ MPP⁺ vs Ringer). In panels B–D histograms represent the total DA output measured as the sum of five (100 min) consecutive samples after the same conditions of A and C (** $p < 0.01$ 6-OHDA (B) and MPP⁺ (D) vs. Ringer). Adapted from Di Matteo et al. (2006a), with permission from Elsevier

can be used as a chronic model of parkinsonism, by allowing a 1-month delay after intranigral MPP⁺ administration, and it was shown that the infusion of MPP⁺ into the SN may cause chronic damage to the nigrostriatal pathway. Therefore, using various DA-ergic toxins and DA releasing agents such as high K⁺ or amphetamine, besides MPP⁺ for the test challenge of the second day, it is possible to employ this method for both the study of neuroprotective or restorative treatments for PD.

Contribution of Microdialysis To Neuroprotective Studies for Parkinson's Disease

As already reported, DA autoxidation and sustained DA turnover can lead to free radical formation, which in turn causes oxidant damage in the iron-enriched nigral neurons during senescence and in PD. Moreover, several compounds have demonstrated their antioxidant properties in preclinical and clinical studies (Di Giovanni 2007, 2008). Monoamine oxidase (MAO) is one of the most important enzymes in neurotransmitter metabolism. Although DA is metabolized by both MAO-A and MAO-B, most therapeutic attention has focused on MAO-B as it is the predominant form in the basal

ganglia, and a number of studies have suggested neuroprotective properties of MAO-B inhibitors. MAO-B regulates both the free intraneuronal concentration of DA and its releasable stores by converting it to its corresponding carboxylic acid DOPAC; therefore, the inhibition of this metabolic pathway prolongs the activity of both endogenously and exogenously derived DA, and thus these drugs are considered specific to the treatment of PD (Riederer et al. 2007). In vivo microdialysis studies of animals models of PD also suggested neuroprotective effects on DA-ergic neurons by the inhibition of MAO activity (Nomoto and Fukuda 1993; Wu et al. 2000). On the other hand, several authors suggested that not only the inhibition of MAO-B by L-deprenil (selegiline) but also other mechanisms may contribute to its neuroprotective actions (Chiueh et al. 1992a,b; Wu et al. 1993; Wachtel and Abercrombie 1994; Matsubara et al. 2001). Indeed, Chiueh et al. (1992a,b) found that selegiline in combination with the MAO-A inhibitor clorgyline inhibited the enhanced formation of $\cdot$ OH induced by the 2'-methyl analog of MPTP. Similarly, Wu et al. (1993) found that pretreatment with selegiline, administered into the striatum, decreased the formation of $\cdot$ OH elicited by the intrastriatal injection of MPP⁺. The enzyme xanthine oxidase (XO) is also thought to be a source of superoxide anion radical that is involved in the generation of $\cdot$ OH species. Thus, allopurinol, an inhibitor of XO suppressed $\cdot$ OH generation induced by

MPP⁺ (Obata et al. 2001b, 2006c). There is considerable evidence that intracellular iron mediates the toxicity of excess of reactive oxygen species, such as superoxide anion, hydrogen peroxide, and hydroxyl free radical to the cells, and it was reported that desferrioxamine, a strong iron (III) chelator, protects the nigrostriatal system against the neurotoxic effect of MPP⁺ (Matarredona et al. 1997; Santiago et al. 1997). Moreover, Obata (2006b) showed that the elevation of DA release by the intrastratial infusion of MPP⁺ in the presence of oxygen and iron may cause $\cdot\text{OH}$ generation through its extracellular auto-oxidation, and this may be counteracted by the local application of iron (III) and not by desferrioxamine. Furthermore, phytic acid, a nutrient contained in high-fiber foods such as cereals and legumes, was found to have antiradical effects by chelating iron for MPP⁺-enhanced $\cdot\text{OH}$ generation via the Fenton-type reaction (Obata 2003). Interestingly, reserpine-induced DA depletion reduced MPP⁺ stimulated $\cdot\text{OH}$ formation in the rat striatum (Obata 1999a), and several microdialysis studies demonstrated the potential neuroprotective properties of DA agonists by scavenging free radicals (Opacka-Juffry et al. 1998; Cassarino et al. 1998; Ferger et al. 2000). Nevertheless, R-apomorphine has been suggested to be neuroprotective because of its effects on presynaptic DA receptors, thereby normalizing DA turnover and therefore free radical formation associated with its metabolism (Yuan et al. 2004). Pergolide in association with L-DOPA was shown to restore striatal DA levels in hemiparkinsonian rats (Dethy et al. 1999) and the coadministration of a DA D₂ receptor agonist such as quinpirole could reduce the excessively high DA levels produced after L-DOPA administration, thereby possibly avoiding further neuronal degeneration (Sarre and Michotte 1996) probably by keeping extracellular levels of DA at more "physiological" levels. In this respect, it was shown that systemic L-DOPA underwent autooxidation in the striatal extracellular compartment of freely moving rats (Serra et al. 2000) promoting nonenzymatic oxidation of released DA, and endogenous melatonin might play an active role in maintaining the oxidative homeostasis in the striatum (Rocchitta et al. 2005, 2006); thus, the coadministration of melatonin at pharmacological doses might be beneficial as adjuvant of L-DOPA therapy (Rocchitta et al. 2006).

It is well-known that angiotensin II converting enzyme (ACE) stimulates striatal DA release and ACE inhibitors (SH group containing captopril and non-SH group-containing enalaprilat or imidaprilat) may suppress DA and $\cdot\text{OH}$ efflux induced by *para*-nonylphenol and MPP⁺, suggesting that antioxidant effects of these drugs probably did not depend only on SH group in their structure (Obata 1999b, 2006d; Obata et al. 2008). It is known that the angiotensin II-induced DA release is Ca²⁺-dependent, and therefore, the antioxidant effect of ACE inhibitors may be also due to the

suppression of Ca²⁺-dependent release of DA (Obata 2002). Indeed, diltiazem, a L-type calcium channel antagonist, suppressed $\cdot\text{OH}$ generation induced by bisphenol A and MPP⁺ (Obata et al. 2002a). As bisphenol A, *para*-nonylphenol is an environmental compound that disrupts various tissues via steroid receptor (Obata et al. 2001b, c), and tamoxifen, a synthetic nonsteroidal antiestrogen, was shown to have antioxidant properties in the rat striatum (Obata and Kubota 2001; Obata 2006e). The blockage of DA oxidation by histidine, a free amino acid with singlet oxygen (¹O₂) scavenger activity, further prevented *para*-nonylphenol-induced $\cdot\text{OH}$ generation in MPP⁺-treated rats (Obata et al. 2001c). Antioxidant activity was found also by nicotine (Obata et al. 2002b) or inhibiting low-density lipoprotein (LDL) oxidation by fluvastatin (Obata and Yamanaka 2000). Pretreatment with sodium salicylate significantly and almost completely protected MPTP-induced striatal DA depletion, locomotor activity, and the loss of nigral DA-ergic neurons (Mohanakumar et al. 2000). Similar protective effects of aspirin were observed in the Parkinsonian model induced by both MPP⁺ and 6-OHDA (Di Matteo et al. 2006a). Sodium salicylate and aspirin had no effects on the uptake of DA and monoamine oxidase activity in the striatum (Mohanakumar et al. 2000; Di Matteo et al. 2006a); therefore, their protective effects against DA-ergic toxins-induced neurotoxicity may be based on the free-radical scavenging activity or other pharmacological properties, but not by their cyclooxygenase (COX)-inhibiting action (Mohanakumar et al. 2000; Di Matteo et al. 2006a). A direct role of NO in the neurotoxic effects of MPTP has been evidenced, since pharmacological inhibition of NOS in the brain rendered neuroprotection against MPTP (Smith et al. 1994; Smith and Bennett 1997; see Obata 2006a for review). Pretreatment with 7-NI or N (G)-nitro-L-arginine methyl ester (L-NAME), inhibitors of NOS, was also reported to be protective against DA-ergic damage induced by in vivo infusion of MPP⁺ in freely moving rats (Rose et al. 1999; Obata and Yamanaka 2001; Di Matteo et al. 2006b) by suppressing toxin-induced $\cdot\text{OH}$ production (Rose et al. 1999; Obata and Yamanaka 2001). The MPP⁺ effect was also completely blocked by the intrastratial perfusion of dizocilpine (MK801) a NMDA ion channel antagonist (Smith and Bennett 1997). Indeed, MPP⁺ may activate NOS by increasing GLU release, which through NMDA channels increases calcium influx (Carboni et al. 1990) and activate constitutive neuronal NOS (Smith and Bennett 1997). A growing body of evidence indicates that an increase of NMDA transmission in the striatum is the major functional consequence of the nigrostriatal DA-ergic degeneration during PD; thus, there are numerous reports regarding the protective effects of the NMDA receptor antagonist MK-801, or NR2A- and NR2B-selective NMDA antagonists, that show reduced undesired side effects with respect to MK-801,

against the loss of nigrostriatal DA (Carboni et al. 1990; Santiago et al. 1992; Richard and Bennett 1995; Smith and Bennett 1997; Marti et al. 2000; Fantin et al. 2008; Sarre et al. 2008). The activation of group II mGlu receptors also produced protective effects against the neurotoxic action of MPP⁺ as a consequence of the release of brain-derived neurotrophic factor (BDNF), a protein with neurotrophic-like properties, capable of activating glial cells that, in turn, show scavenger activity and tissue repair (Matarredona et al. 2001). Furthermore, antagonism at mGlu 5 receptors provided specific protection against dyskinesia, due to an excessive responsiveness of *direct* pathway neurons to L-DOPA, through normalization of GABA overflow in the SNr of a rat model of PD (Mela et al. 2007). There is also evidence that the coadministration of NMDA receptor antagonists in conjunction with L-DOPA has seen some success (Jonkers et al. 2000, 2002; Arai et al. 2003; Sarre et al. 2008) normalizing excessive striatal DA and GLU release due to L-DOPA, cause of dyskinesias and “on-off” phenomena (Jonkers et al. 2000, 2002). Because L-DOPA is not able *per se* to induce dyskinesia or motor fluctuations since these side effects are due to an unregulated DA efflux into the striatal extracellular space, consequent to the lack of high-affinity reuptake by the DA plasma membrane transporter and by missing nigrostriatal autoreceptors for the regulation of synthesis and DA release that predispose degenerated neurons to these side effects; thus, a physiological balance of DA transmission could not be achieved leading to the high fluctuations of extracellular DA levels induced after repeated L-DOPA (Abercrombie et al. 1990; Wachtel and Abercrombie 1994; Maeda et al. 1999; Miller and Abercrombie 1999; Meissner et al. 2006; Rodriguez et al. 2007; Buck and Ferger 2008). The inhibition of the enzyme aromatic amino acid decarboxylase, which converts L-DOPA to DA, by benserazide was also able to prevent or reduce these undesired side effects, when coadministered with L-DOPA, by inhibiting the rapid changes of extracellular DA concentration due to the administration of L-DOPA alone (Jonkers et al. 2001; Shen et al. 2003; Buck and Ferger 2008). In a series of studies it was demonstrated that exogenous L-DOPA-derived DA is mainly stored in and released from serotonergic neurons when nigrostriatal neurons are denervated (Tanaka et al. 1999; Kannari et al. 2000, 2001). Also, fluoxetine attenuated L-DOPA-derived extracellular DA levels in the striatum of 6-OHDA lesioned rats, due to its indirect 5-HT_{1A} agonistic property (Yamato et al. 2001), as demonstrated also by another study that showed stimulation of 5-HT_{1A} receptors reduced an increase in extracellular DA derived from exogenous L-DOPA (Kannari et al. 2001). Interestingly, the 5-HT_{1A} agonist R-(+)-8-OHDPAT also decreased extracellular GLU and aspartate in DA-denervated striatum of rats, showing anti-Parkinsonian efficacy (Mignon and Wolf 2005). Further, other serotonergic

agent such as 5-HT_{2A/2C} receptor antagonists may provide an attractive non DA-ergic target for improving PD therapies (Nowak et al. 2006; see Di Giovanni et al. 2008 for review). The reduction of GLU release in the SNr may represent the mechanism by which nociceptin/orphanin FQ (NOP) receptor antagonists reverse parkinsonism, because this class of compounds normalized haloperidol-evoked GLU levels in the SNr, an effect that correlated with attenuation of akinesia (Marti et al. 2004b, 2005), whereas NOP receptor antagonists elevate SNr GABA release (Marti et al. 2007) and nigrostriatal DA transmission (Marti et al. 2004a). The combined administration of a NOP receptor antagonist (J-113397) and L-DOPA produced additive attenuation of Parkinsonian-like symptoms through an increase in SNr GABA release and consequent over-inhibition of SNr GABAergic neurons projecting to the ventromedial thalamus, leading to the disinhibition of thalamocortical glutamatergic projections and locomotion (Marti et al. 2007). This appears relevant also in humans, in which motor improvement induced by deep brain stimulation in the subthalamic nucleus was associated with lowered GABA release in the motor thalamus (see next section, Stefani et al. 2005, 2006; Galati et al. 2006).

As already discussed, the balance of DA, GABA, and GLU is critical to maintaining the normal function of basal ganglia. It is important to remember that the degeneration of DA-ergic neurons in the SNc results in a decreased DA-ergic activity in the striatum while increasing glutamatergic activity in the STN. This overactivity can lead to an increased GLU-mediated excitation in basal ganglia output regions, such as the SNr and GPi, which may in turn lead to a reduced thalamo-cortical feedback (Fig.1) and the subsequent appearance of akinesia (Blandini et al. 2000). Therefore, enhancing the activity of DA in *direct* pathway and reducing the activity of GLU in *indirect* pathway have been proposed as two therapeutic strategies for PD (Blandini et al. 2000; Bianchi et al. 2003). In this respect, iptacalim, a novel ATP-sensitive potassium channel opener, inhibited both the increase of extracellular GLU and the decrease of extracellular DA in the lesioned-side striatum of rats (Yang et al. 2006a,b; Wang et al. 2006). Furthermore, systemic KW-6002, an adenosine_{2A} (A_{2A}) receptor selective antagonist, increased both GABA and GLU in the SNr (Ochi et al. 2004a). Since the A_{2A} receptor antagonism decreased pallidal (GPe) GABA concentrations in 6-OHDA lesioned rats (Ochi et al. 2000), this decrease might induce the disinhibition of pallidal GABA-ergic neurons, in turn increasing nigral GABA levels via GABA-ergic neurons from the GPe to SNr, thus resulting in the stimulation of thalamic glutamatergic projections to the cortex and therefore to the striatum (Ochi et al. 2000, 2004a; Corsi et al. 2003). On the other hand, an increase of nigral GLU release by KW-6002 would be apparently inconsistent with the current standard model of basal ganglia pathophysiology (Albin et al. 1989; DeLong

1990). However, it is consistent with recent analyses of deep brain stimulation effects that are accompanied by an increased release of GLU in the SNr (Windels et al. 2000; Boulet et al. 2006) and recent results that show an increase of nigral GLU release by L-DOPA (Ochi et al. 2004b). An interesting possibility linked to the role of A_{2A} receptors in control of movement in PD is the potential modification of the A_{2A} receptor. As suggested by Antonelli et al. (2006), in 6-OHDA lesioned rats, the presence of an abnormal increase in A_{2A} signaling, which then prevails over D₂ signaling in the control of GABA release in GP, might diminish the therapeutic efficacy of L-DOPA at D₂ receptor site; therefore, the blockade of the A_{2A} receptor prevailing tone could be one of the factors underlying the positive effects produced by A_{2A} antagonists in PD (Tanganelli et al. 2004; Antonelli et al. 2006).

High-frequency stimulation of the subthalamic nucleus (STN-HFS) has emerged as a powerful therapeutic approach for the treatment of PD patients, and it has been suggested that STN-HFS acts by increasing striatal DA release, tyrosine hydroxylase (TH) activity, and DA metabolism (Bruet et al. 2001; Meissner et al. 2001, 2002, 2003). Interestingly, in DA-depleted rats, STN-HFS stabilized the L-DOPA-induced increase of striatal DA levels (Lacombe et al. 2007), probably by modulating DA turnover, uptake, and synthesis, thus suggesting that adaptive mechanisms, such as the restoration of autoregulation for presynaptic DA release in the striatum, are involved in the stabilization of striatal DA concentrations potentially involved in alleviating L-DOPA-related motor fluctuations (Nimura et al. 2005; Lacombe et al. 2007). STN-HFS also increases striatal GLU levels in both normal and hemiparkinsonian rats, suggesting an increase in the activity of striatal excitatory glutamatergic afferents (Bruet et al. 2003). Indeed, the inhibition of basal ganglial output activity by STN-HFS (Windels et al. 2005; Boulet et al. 2006) may overcome the inhibition of the thalamocortical pathway activating bilateral corticostriatal projections via cortical collaterals, and probably, also the thalamostriatal pathway, as shown by the increase of extracellular GABA levels in the SNr after STN-HFS in lesioned rats (Windels et al. 2005; Boulet et al. 2006). Also microdialysis and behavioral studies showed that GABA-ergic tone in SNr plays a role in the regulation of motor functions of the basal ganglia. Indeed, the stimulation of locomotor activity and reduction of tremulous jaw movements, which have many of the characteristics of PD tremor, were linked to an increase in SNr GABA release (Morari et al. 1996; Trevitt et al. 2002; Ishiwari et al. 2004).

Although current therapies for PD focus on treating the symptoms of the disorder, neurotrophic factors offer the promise of preventing, delaying, or even reversing the loss of the DA neurons themselves. The potent DA-ergic trophic factor glial cell line-derived neurotrophic factor (GDNF)

offers a promising approach to potentially repairing and restoring function to damaged DA-ergic neurons. Thus, both the induction of endogenous GDNF with long-term 1,25-dihydroxyvitamin D₃ (calcitriol) treatment (Smith et al. 2006) and the injection of vector coding for GDNF protein (Gerin 2002; Smith et al. 2005), or exogenous GDNF into the nigra of 6-OHDA-lesioned rats (Opacka-Juffry et al. 1995; Hoffman et al. 1997) and aged or lesioned monkeys (Grondin et al. 2003a; Gerhardt et al. 1999) lead to similar changes in nigrostriatal DA neuron functioning, resulting in an increase of extracellular DA and improved motoric function.

Cellular therapy with stem cells also appears to be an opportunity to restore the DA-ergic function in PD. Indeed, the perfusate from the grafted striatum with mesencephalic DA-ergic neurons showed levels of DA, DOPAC, and HVA that were not statistically different from those of the intact contralateral striatum (Zetterström et al. 1986; Strecker et al. 1987; Rioux et al. 1991; Kondoh and Low 1994). In addition, the response of DA released from these grafted DA neurons to apomorphine and nomifensine suggests that the DA-receptor-mediated autoregulatory control and the normal DA reuptake mechanism were also functioning in these cells (Strecker et al. 1987), and the rise of DA release by amphetamine or dihydrokainic acid further indicates that grafted DA neurons became functionally integrated into the host circuitry (Zetterström et al. 1986; Rioux et al. 1991; Kondoh and Low 1994). More recent studies also demonstrated that embryonic mouse-derived DA-ergic stem cells (ESC) or rat adult bone-marrow mesenchymal stem cells (MSC), grafted into the DA-depleted striatum, may survive and ameliorate amphetamine-induced ipsilateral rotation for a long period of time. Moreover, this functional recovery was correlated to increased extracellular levels of DA and its metabolites in the grafted striatum, as determined by microdialysis (Rodríguez-Gómez et al. 2007; Bouchez et al. 2008), as well as increased DA release in response to pharmacological challenges in vivo, including K⁺-induced depolarization, nomifensine-induced inhibition of DA reuptake and amphetamine-induced DA release (Rodríguez-Gómez et al. 2007) and to an increased density of DA-ergic markers such as TH, DA transporter (DAT), and vesicular monoamine transporter 2 (VMAT₂) (Bouchez et al. 2008), suggesting that these cells show DA release and reuptake and stimulate appropriate postsynaptic responses for long periods after implantation.

Microdialysis in Parkinsonian Patients

Microdialysis in the brain of PD patients was performed for the first time in 1989 (Meyerson et al. 1990) during neurosurgical thalamotomy to relieve tremor. This was possible as the microdialysis probes were introduced through the same

trajectory as the lesioning electrode, thus causing no additional damage to the brain. In this study, DA, DOPAC, HVA, 5-hydroxyindoleacetic acid (5-HIAA), GABA, GLU, and other neurotransmitters were measured in the dialysates from the thalamus. The results showed that the technique was safe; no additional damage was observed; and the levels of neurotransmitters were reproducible after a short period of stabilization. Later, a significant decrease of extracellular GABA in the thalamus both during high-frequency electrical stimulation and following a thermolesion of the thalamus was found (Rada et al. 1999). This technique was also used during neurosurgery of patients with PD implanted for deep brain stimulation (DBS) in the GPi and STN, after prolonged therapy washout (Fedele et al. 2001a,b). In these studies, the extracellular levels of aspartate, GLU, glycine, and GABA in the GPe, GPi, and STN were measured before (off-state) and after (on-state) the perioperative administration of a clinically effective dose of apomorphine. In the “off-state” was found that GABA basal levels in the GPi were significantly higher than those measured in the GPe and STN, in contrast with the classic pathophysiological model of PD, as both the GABA-ergic pathways reaching this brain region (the *direct* pathway from the putamen and the *indirect* pathway via collaterals of the GPe fibers directed to the STN) are supposed to be less active in “off-state” PD patients (Albin et al. 1989; DeLong 1990). Therefore, these findings indicate that the GPi receives more GABA-ergic inputs than the other basal ganglia nuclei and could represent a biochemical marker for GPi target identification in PD surgery (Fedele et al. 2001a,b). Despite the expected microdialysis, changes due to the apomorphine-induced stimulation of DA-ergic receptors would be a decrease of GLU in the GPi (and possibly in the GPe) and an increase of GABA in the STN (and possibly in the GPi). However, its administration failed to alter amino acid levels in all the brain structures investigated, even though it was indeed efficacious at brain nuclei level on clinical and electrophysiological variables, thus suggesting that the acute effects of the DA-ergic therapy are not due to the modulation of neuroactive amino acid release. As the authors themselves acknowledged, such negative findings may be due to the relatively low dose of apomorphine used in the study for safety reasons. In fact, rather high doses (10 to 100 times higher than those used in that study) are used in most of the animal studies, where apomorphine effects have been observed. Moreover, the regulation of aspartate, GLU, glycine, and/or GABA neuronal release by apomorphine might be obscured by the high extracellular levels of the nonneuronal pool of these amino acids. Recently, to unravel the mechanisms underlying the beneficial effects of DBS of the STN (STN-DBS), the same research group has investigated the effects of this type of stimulation on the extracellular levels of cGMP, as a marker of glutamatergic

transmission, in the GPi, SNr, and putamen of PD patients, and GABA levels in the anteroventral thalamus (VA) (Stefani et al. 2005, 2006; Galati et al. 2006). These authors found that extracellular cyclic GMP basal levels were significantly augmented in the GPi, SNr, and putamen during clinically effective STN-DBS, whereas extracellular GABA levels in the anteroventral thalamus (VA) were decreased, indicating that DBS differentially affects fibers crossing the STN area: it activates the STN-GPi/SNr pathway while inhibiting the GPi-VA. These findings support a thalamic disinhibition that, in turn, reestablishes a more physiological level of putamen activity, as the main factor responsible for the clinical effect of STN-DBS in Parkinsonian patients.

Conclusions

The current problem for treating PD is in the lack of therapies that can provide a long-term benefit in the absence of disabling complications. Despite the success obtained with animal models, clinical neuroprotection is much more difficult to accomplish (Di Giovanni 2008). More sensitive reliable methods and clinical correlative markers are required to discern between confoundable symptomatic effects and a possible neuroprotective action of drugs, namely, the ability to delay or forestall disease progression by protecting or rescuing the remaining DA neurons or even restoring those that have been lost. The ideal drug for treating PD remains to be discovered and the current animal models are usually models of an acute DA-ergic deficit, although more chronic intoxication protocols have been developed. On the other hand, at present, there is no evidence to show that the chronic administration of low dose of any toxic substance mimics the progressive degenerative nature of PD, since PD is a multiple system disorder and the degenerative process is not limited to the DA-ergic nigrostriatal pathway. Indeed, other cell populations containing different neurotransmitters, such as the noradrenergic locus ceruleus, the serotonergic raphe nucleus, the cholinergic and glutamatergic pedunculopontine nuclei, and the cholinergic nucleus of Meynert are also affected, and other factors such as genetic and environmental events influence the pathogenesis of PD and these degenerative changes are not reproduced in current experimental models (Linazasoro 2004). Furthermore, it is important to better define the function of the basal ganglia in health and disease for devising novel efficacious treatments for PD. Ideally, new therapeutic options can be classified into three main categories:

- (a) Neuroprotective treatments, which aim at halting the neurodegenerative process at the basis of PD. For

example, investigators are attempting to counteract the degeneration of DA neurons through intracerebral delivery of trophic factors (Gill et al. 2003; Grondin et al. 2003a,b) or systemic administration of antioxidants (Shults et al. 2002; Mandel et al. 2003; Ramassamy 2006).

- (b) Restorative treatments, which aim at restoring the integrity of the damaged nigrostriatal system by intracerebral transplant of DA-producing cells (Lindvall and Hagell 2000; Björklund et al. 2003) or by delivery of viral vectors encoding DA-synthesizing enzymes (Kirik et al. 2002).
- (c) Symptomatic treatments, which aim at improving the symptoms of the disease or the motor complications that result from traditional L-3,4-dihydroxyphenylalanine (L-DOPA) pharmacotherapy. Different animal models, including those discussed herein, may be best suited to these different applications. Hopefully, the ideal model for testing the efficacy of neuroprotective treatments would be one where the etiopathological features and time course of DA cell degeneration closely resemble the events that occur in PD.

Since the microdialysis approach has largely contributed to this field, it may further help in the future to obtain a better understanding of the disease and to develop novel therapeutic strategies for PD both in preclinical and clinical studies.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 19

Inflammatory Response in Parkinsonism

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Abstract Inflammatory responses have been proposed as important factors in dopaminergic neuro-degeneration in Parkinsonism. Increasing evidence suggests that the alteration of the glial microenvironment induced by neuronal degeneration could be deleterious to the remaining neurons. The activation of microglia/macrophages and reactive astrocytes may have a negative effect on the surrounding parenchyma, perpetuating the neurodegenerative process. However, this alteration may also go beyond the brain parenchyma and stimulate other inflammatory changes in other systems, inducing the release of proinflammatory cytokines and probably Acute Phase Proteins (APP) and Glucocorticoids (GC). In this work we review the latest advances in the field to provide a picture of the state of the art of studies of inflammatory responses and Parkinsonism, hopefully opening up new therapeutic perspectives for patients with Parkinson's disease.

Keywords Microglia • Astroglia • Neuroinflammation • Anti-inflammatory drugs • Parkinson's disease • Dopaminergic degeneration

Introduction

Patients with Parkinson's disease (PD) show alterations of inflammatory markers. The analysis of blood serum and cerebrospinal fluid (CSF) obtained from PD patients points to an increase in inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) (Mogi et al. 1994a), interleukin (IL)-1 beta, IL-2, IL-4, IL-6 (Blum-Degen et al. 1995; Mogi et al. 1996; Stypula et al. 1996), and interferon γ

(IFN- γ) (Mount et al. 2007). Postmortem biochemical analysis of brains from patients dying with PD shows that some of those cytokines are also increased in the nigrostriatal system (Mogi et al. 1994a, b). This particular inflammatory reaction observed inside and outside the brain parenchyma may be caused by a glial cell reaction as a result of dopaminergic degeneration (Hirsch et al. 1998; Nagatsu and Sawada 2007). Indeed, the most evident degenerating region of PD patients, the Substantia Nigra *pars compacta* (SNpc), shows large numbers of activated microglia (McGeer et al. 1988) and reactive astrocytes (Forno et al. 1992). These two cell types are presumably responsible for the increase of these cytokines in the nigro-striatal pathway and probably also outside the brain parenchyma, since they are able to produce and release cytokines (Tedeschi et al. 1986; Dickson et al. 1993; Benveniste et al. 1994; Hanisch 2002). In turn, these released cytokines may also activate the surrounding microglia and astrocytes, but importantly, may also act on neurons. Moreover, it has been established that dopaminergic neurons display the TNF- α receptor, which suggests that local inflammation could induce neuronal degeneration in the SNpc (Boka et al. 1994). It is not clear whether the inflammatory response is a cause or a consequence of dopaminergic cell loss and it is not known whether the release of inflammatory factors is part of a harmful or protective mechanism toward the dopaminergic neurons (Fig. 1 and Table 1). Experimental results suggest that it is most probably a consequence, but much research in this field is still necessary to clarify this issue.

Some epidemiological studies have shown that people who regularly use antiinflammatory drugs have less risk of developing clinical PD (Chen et al. 2003, 2005). However, other studies only found a slight trend (Bower et al. 2006) or even no relation between the use of nonsteroidal antiinflammatory drugs and PD (Ton et al. 2006; Bornebroek et al. 2007; Hancock et al. 2007).

Experimental data suggest that antiinflammatory drugs could be a promising therapy, at least to slow down the progression of the associated degeneration or diminish the local harmful; however it is still not clear whether or not

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Fig. 1 Inflammatory response in the brain parenchyma. Dopaminergic cell death occurring in the brain parenchyma induces activation of glial cells (astrocytes and microglia). Glial activation is characterized by the release of cytokines and growth factors which can reactivate other surrounding glial cells and induce new events of neuronal loss. Growing factors may stimulate the formation of new blood vessels and cytokines may cross the BBB, probably recruiting blood cells into the brain parenchyma

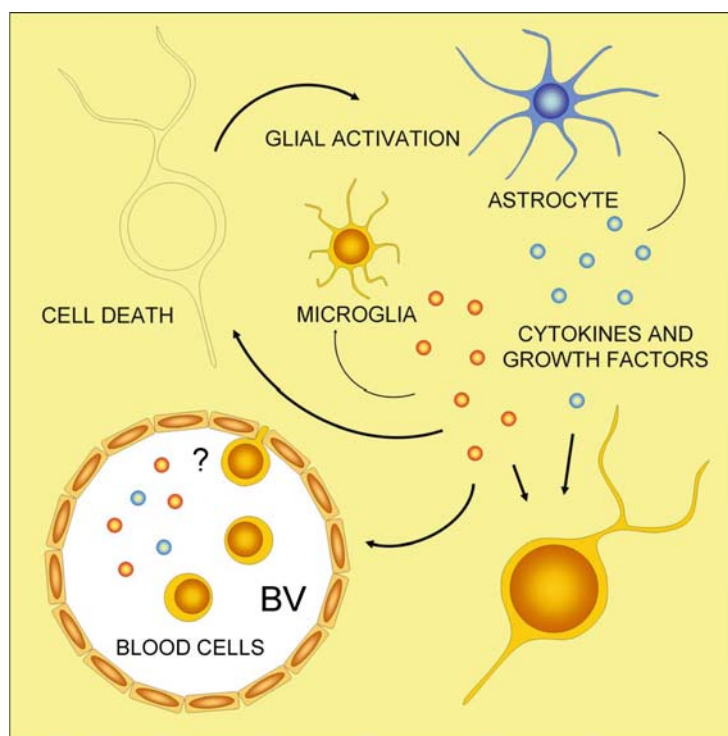


Table 1 Summary of some of the inflammatory factors involved in Parkinsonism

	PD	EP	Knock-out mice
TNF- α	$\uparrow\uparrow$	$\uparrow\uparrow$	–
IFN- γ	$\uparrow\uparrow$	$\uparrow\uparrow$	Protected against MPTP Mount et al. (2007)
IL-1 β	$\uparrow\uparrow$	$\leftrightarrow$ Barcia et al. (2005b)	–
TNF- α R	–	–	Protected against MPTP Sriram et al. (2002)
COX-1	No protection?	No protection	Non protected Feng et al. (2003)
COX-2	Protection?	Protection	Protected against MPTP Feng et al. (2002)
GR	?	?	Increased vulnerability to MPTP Morale et al. (2004)
Dexamethasone	?	Protection Kurkowska-Jastrzebska et al. (1999)	–
17- β Estradiol	–	Protection Callier et al. (2001)	–
Progesterone	–	Protection Callier et al. (2001)	–
Cortisol	$\sim$	CR Mizobuchi et al. (1993); Barcia et al. (2003b)	–
Adenocorticotropin	$\sim$ Rabey et al. (1990)	$\sim$ Hinenio et al. (1992) and Mizobuchi et al. (1993)	–
CRP	$\uparrow\uparrow$ McGeer and McGeer (2004)	$\uparrow\uparrow$ de Pablos et al. (2004)	–

Abbreviations: PD Parkinson's disease, EP experimental Parkinsonism, $\uparrow\uparrow$ increased levels, $\sim$ alteration, – no data, ? unknown, CR conflicting results

these common drugs may also be beneficial to those individuals who already have PD (Esposito et al. 2007).

The Story of MPTP

It is well known that in the early 1980s a group of young drug addicts was intoxicated with 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) in the streets of Santa Clara,

California, developing chronic Parkinsonism as a result (Langston et al. 1983). This discovery provided an excellent tool for experimental Parkinsonism over the years. However, it was not until the late 1990s that a postmortem analysis was made of three of the deceased (Langston et al. 1999). In this study, Langston and coworkers reported a massive neuronal degeneration in the SNpc as was expected, but importantly, they also described the presence of activated microglia as was previously reported in PD patients (McGeer et al. 1988).

This finding suggested that dopaminergic nerve degeneration could be activated and perpetuated over the years following a single neurotoxic insult, involving inflammatory factors. In this scenario, microglial cells would be activated by an initial dopaminergic neuronal loss and such an activation would induce new cytokine-mediated degeneration in the remaining neurons. Since all the patients were treated with L-DOPA or dopaminergic agonists for several years, some researchers suggested that either treatment could have been responsible of the inflammatory reaction. However, other reports demonstrated that inflammatory response also appears in the absence of L-DOPA and does not have an effect in neuronal degeneration (Murer et al. 1998; Ferrario et al. 2003; Barcia et al. 2004b).

Experimental data have been crucial for understanding this inflammatory process in Parkinsonism. For example, mice and monkeys treated with MPTP also show activation of the microglial cells associated with dopaminergic cell loss (Czlonkowska et al. 1996; McGeer et al. 2003; Barcia et al. 2004b). Importantly, studies performed in monkeys provided a significant finding when microglial activation was seen to occur several years after the last MPTP injection and independently of L-DOPA treatment (McGeer et al. 2003; Barcia et al. 2004b). All these data support the idea that the inflammatory reaction, mediated by microglial cells, might be responsible for the perpetuation of neuronal degeneration in the SNpc.

These data have encouraged many researchers to test different antiinflammatory drugs in induced Parkinsonian animals. Indeed, many compounds have been proved to protect against dopaminergic degeneration in experimental-induced Parkinsonism and their use could represent a potential therapy for Parkinsonian patients (McGeer and McGeer 2007; Tansey et al. 2007).

Glucocorticoids

Glucocorticoids, such as dexamethasone, protect dopaminergic neurons against MPTP-induced degeneration in mice (Kurkowska-Jastrzebska et al. 1999, 2004). The mechanisms by which glucocorticoids are able to protect against neurodegeneration are poorly understood. However, the study of other steroid hormones has helped to understand their antiinflammatory role and their possible protective function toward dopaminergic neurons. Steroid hormones, such as 17 β -estradiol or progesterone, protect against induced dopaminergic degeneration (Dluzen et al. 1996; Callier et al. 2001; Ramirez et al. 2003; Morale et al. 2006), which could explain the slightly higher prevalence of PD in men than in women (Wooten et al. 2004) (Fig. 2). The action of estrogens may reduce the inflammatory processes occurring in the brain

parenchyma, diminishing the activation of the glial cells surrounding dopaminergic neurons (Tripanichkul et al. 2006), a process possibly mediated by the differential expression of estrogen receptors in glial cells and neurons (Bains et al. 2007). In fact, the absence of estrogen receptors results in high susceptibility to MPTP-induced dopaminergic neurodegeneration (Morissette et al. 2007) and similar results have been demonstrated in mice lacking the glucocorticoid receptor (Morale et al. 2004). Importantly, these knockout animals presented an iNOS-exacerbated response in the activated microglia after MPTP treatment, which suggests that glucocorticoids may function by inactivating the glial response (Morale et al. 2004). However, very little is known about how glucocorticoids act on dopaminergic neurons. Dopaminergic neurons present glucocorticoid receptors and their activation may induce a protective response. Recent, ongoing experiments in our group strongly suggest that the expression of the glucocorticoid receptor may protect dopaminergic neurons after MPTP treatment in nonhuman primates (unpublished results) (Fig. 3).

Some PD patients show alterations at the hypothalamic hypophyseal adrenal level (Rabey et al. 1990), and dopaminergic neuronal loss may induce increased levels of glucocorticoids as a neuro-protective mechanism. It has been reported that weeks after MPTP administration, adrenocorticotrophin and cortisol concentrations increase and their circadian rhythm are altered (Hineno et al. 1992; Mizobuchi et al. 1993). However, one year after MPTP administration, these changes were undetectable in monkeys despite the still-active local inflammation in the SNpc (Barcia et al. 2003b). Taken together, these results suggest that glucocorticoids may play an important role in dopaminergic degeneration and may represent a potential therapy for PD.

Nonsteroidal Antiinflammatory Drugs

Since glucocorticoid administration may provoke undesirable side effects, the search for nonsteroidal antiinflammatory drugs for Parkinson's disease treatment has been prolific. Countless products have been tested in experimental Parkinsonism and many of them seem to be neuroprotective and appear to offer potential therapy (Schiess 2003; Chen et al. 2005; Bornebroek et al. 2007; Wahner et al. 2007). Drugs, such as aspirin or ibuprofen, protect against induced dopaminergic degeneration (Aubin et al. 1998; Casper et al. 2000). Nonsteroidal antiinflammatory drugs act by inhibiting the enzyme cyclooxygenase (COX)-1 or COX-2. Aspirin inhibits both COX-1 and COX-2, while others like ibuprofen or meloxicam inhibit only COX-2 (Teismann and Ferger 2001). The use of COX-2 inhibitors would be preferable, since COX-1 is dependent on the prostaglandin system and

Fig. 2 Role of cytokines and glucocorticoids in dopaminergic degeneration. MPTP or an unknown insult (?) is able to induce cell death in dopaminergic neurons. This degeneration induces microglial activation characterized by cytokine release. Cytokine release may induce dopaminergic cell loss but also induces glucocorticoid release, which may protect dopaminergic neurons and inhibit microglial activation

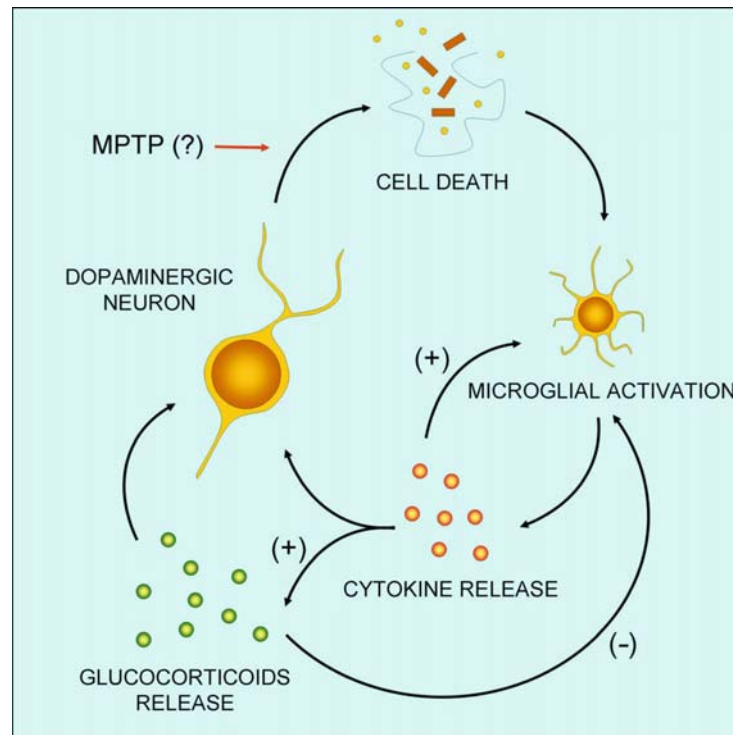
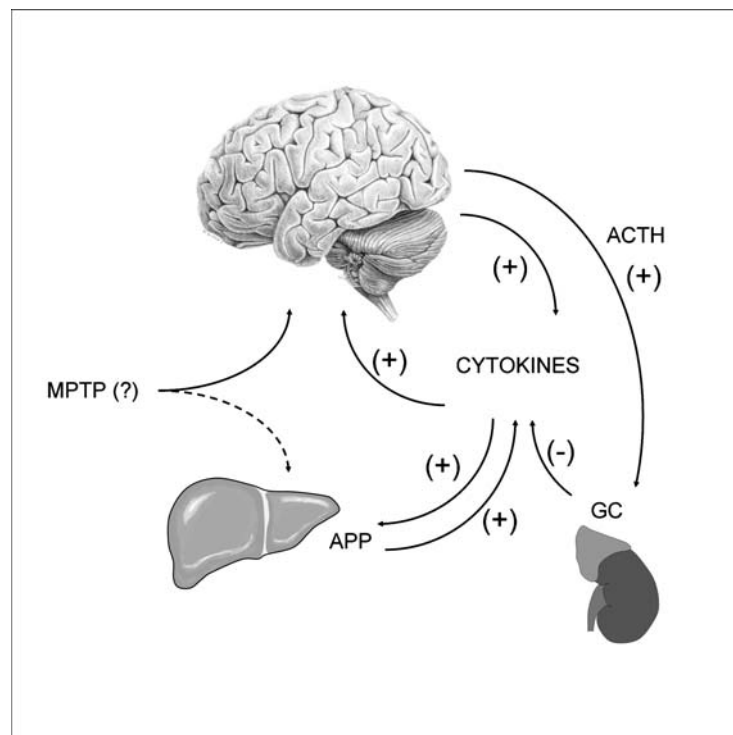


Fig. 3 Generalized inflammatory response in Parkinsonism. MPTP (or an unknown insult) affects the brain (and probably the liver), causing an inflammatory response, characterized by microglial reaction and release of cytokines, which may activate the release of acute phase proteins (APP) in the liver. This inflammatory response may alter ACTH production and stimulate the production and release of glucocorticoids, such as cortisol, in the suprarenal gland. The release of glucocorticoids may inhibit the release of cytokines and may protect neurons from degeneration



its inhibition may cause gastric mucosa damage. In the case of experimental Parkinsonism, it has been established that COX-2, but not COX-1, is specifically involved in dopaminergic degeneration (Feng et al. 2002; Teismann et al. 2003;

Carrasco et al. 2005). Mice lacking COX-2 are protected against MPTP-induced neurodegeneration, while COX-1 knockout animals present similar susceptibility to wild-type animals (Feng et al. 2002, 2003). Importantly, some studies

have demonstrated that COX-2 inhibitors specifically inhibit microglial activation but not astrocyte activation in experimental Parkinsonism (Sanchez-Pernaute et al. 2004), which suggests that microglia could be the final cell type responsible for inflammatory-induced neuro-degeneration. According to these experimental results, microglial-mediated inflammation may induce neuronal degeneration through COX-2 pathways. Although what happens in the disease is not fully understood, according to this, the presence in patients of polymorphisms of proinflammatory genes, such as COX-2 genes, could well provide a genetic predisposition to create an exacerbated microglial activation (Hakansson et al. 2007), which may also be involved in alpha synuclein accumulation in dopaminergic neurons and facilitate a progressive degeneration (Chae et al. 2008; Hirohata et al. 2008). In summary, much evidence suggests that the specific inhibition of COX-2 could be beneficial to stopping or at least diminishing the dopaminergic neurodegeneration and might be regarded as a possible candidate for therapy in PD.

Cytokines

The release of proinflammatory cytokines, like TNF- α and IFN- γ , is usually related to degeneration and tissue damage (Merrill and Benveniste 1996). PD patients show increased levels of cytokines in the brain, blood, and CSF (Mogi et al. 1994a, b; Mogi et al. 1996; Blum-Degen et al. 1995; Stypula et al. 1996; Mount et al. 2007), which may be a cause/effect of the dopaminergic degeneration. Neuronal loss may stimulate the release of cytokines to the surrounding microenvironment, but at the same time, cytokines may also induce dopaminergic cell loss, which could explain the perpetuation of the neurodegenerative process (Barcia et al. 2003a; Hirsch et al. 2003). Glial cells and lymphocytes are responsible for the production and release of cytokines. Both astroglia and microglia are clearly involved in neuronal degeneration in Parkinsonism but the role of lymphocytes in dopaminergic degeneration remains unclear. An increase of lymphocyte infiltration in the SNpc of Parkinsonian patients has been recently reported (Brochard et al. 2009) and similar results have been noted in MPTP-treated mice (Kurkowska-Jastrzebska et al. 1999). However, the persistence of this lymphocyte infiltration is not evident in experimental parkinsonism in mice (Brochard et al. 2009) and recent unpublished results of our group show no infiltration of T cells in the SNpc in rendered chronic Parkinsonian monkeys, which strongly suggests that glial cells, especially microglia, are the only cells responsible for this cytokine release. Results obtained in long-term Parkinsonian monkeys demonstrate that cytokines can be released a long time after dopaminergic degeneration (Barcia et al. 2005b), a release which correlates with dopaminergic degeneration and with microglial activation

in the SNpc (unpublished results). Other experimental results, performed in knockout animals and cell cultures also support the hypothesis that microglia and the release of cytokines, such as TNF- α and IFN- γ , may be responsible for neuronal degeneration in Parkinsonism (Sriram et al. 2006; Mount et al. 2007). Blocking the action of TNF- α in cell cultures protects against dopaminergic neuronal loss, and mice lacking the TNF- α receptor are protected against MPTP-induced degeneration (Sriram et al. 2002; McCoy et al. 2006; Sriram et al. 2006). In the same way, IFN- γ knockout mice are less susceptible to MPTP-induced neuronal loss and it has been demonstrated that dopaminergic neuronal loss in cell cultures is IFN- γ mediated (Mount et al. 2007). Both the TNF- α and IFN- γ released by microglial cells may intervene in dopaminergic cell death in Parkinsonism; however, it is still not known what causes the increased levels of cytokines in PD. Probably, an initial insult to dopaminergic neurons could initiate the inflammatory reaction and the cytokine cascade. Another possible trigger could be the genetic background of the patients. Some studies have described alterations in cytokine-encoding genes in PD patients, which could facilitate the initiation of proinflammatory processes (Hakansson et al. 2005; Bialecka et al. 2008). These facts suggest that glial-released TNF- α and IFN- γ may mediate neurodegeneration in Parkinsonism and indicate that antiinflammatory drugs targeting proinflammatory cytokines may be beneficial to PD therapy.

A Timedependent Generalized Inflammatory Response

Patients show a progressive degeneration of the nigral system. The age of onset seems to be critical to the duration of the disease (Jankovic and Kapadia 2001). Early-onset patients show a strong response to L-DOPA and are more likely to suffer a severe loss of dopaminergic nigral neurons, a longer disease course, and slower rate of disease progression (Kempster et al. 2007). Local inflammation may be responsible for nigral degeneration, but age could also be important for the degree of this response. Recent experiments performed in monkeys have demonstrated that elderly monkeys show higher rates of microglial activation in the SNpc than younger monkeys after MPTP-exposure (Kanaan et al. 2008). This could explain the fact that late onset of the disease involves a faster progression than early onset. Accordingly, recent results of our group have demonstrated that microglial activation increases with time after MPTP insult and that the activation is correlated with cytokines levels and the degree of dopaminergic degeneration, which could explain the nonlinear or exponential time relationship of the deterioration of patients during the late phases of the

disease. It could be that the protective response is decreased with age or that the neurons are more vulnerable with aging.

It is uncertain whether the inflammatory factors are a cause or effect of dopaminergic cell loss in Parkinsonism. It is likely that the inflammatory response is initially protective, activating compensatory mechanisms, and is only harmful when a certain threshold of neuronal loss is crossed. With such an unbalanced response, dopaminergic cell loss could locally activate glial cells and induce the release of cytokines harmful to the remaining neurons. In addition, cytokines are also able to cross the blood–brain barrier (BBB) and send signals to other organs or systems. Cytokines stimulate other inflammatory factors related to cell infiltration and blood vessel formation, such as monocyte chemotactic protein-1 (MCP-1) or vascular endothelium growth factor (VEGF). In fact, the infiltration of T cells in the brain parenchyma of Parkinsonian patients and animals rendered Parkinsonian has been recently demonstrated (Brochard et al. 2009) and an increase of endothelial cells in the SNpc of patients with PD has also been reported (Faucheux et al. 1999), which suggests that neuronal loss may induce blood vessel formation and blood cells infiltration as a result of the inflammatory reaction. Accordingly, studies performed in chronic Parkinsonian monkeys have demonstrated an increase in blood vessels together with an increase in VEGF cells in the SNpc (Barcia et al. 2005a). The significance of this increase of blood vessels in the SNpc is not well known, but it may increase the input of nutrients, growing factors, or other compounds to the brain parenchyma, or it may indicate an alteration of the BBB, which may or may not be beneficial to the remaining neurons (Faucheux et al. 1999; Barcia et al. 2004a). These results imply that the inflammatory response is not only localized in the brain parenchyma but also goes beyond the BBB. Besides the alterations detected in blood, an increase of acute phase proteins, which are mainly synthesized in the liver, has also been noted, more specifically, C-reactive protein (CRP) is increased in brains from patients with PD (McGeer and McGeer 2004). It is not known whether cells infiltrated in the brain parenchyma are responsible for this increase of CRP or whether the protein is traveling from the liver to the area of tissue damage, or both. Our group has recently reported an increase of several acute phase proteins (APP) levels, such as CRP, serum amyloid A, and haptoglobin in serum, after MPTP exposure in nonhuman primates (de Pablos et al. 2004). APP levels reach their highest values immediately after MPTP injection and then decrease gradually to normal levels, which could explain why PD patients show an increase of CRP in the brain but not significant increase in plasma levels (Chen et al. 2008). APPs may increase after dopaminergic cell death events and then decrease in plasma and remain localized only in the brain parenchyma (McGeer and McGeer 2004; Chen et al. 2008).

Parkinsonism is therefore accompanied by the release of cytokines, glucocorticoids, and APP, which imply that the inflammatory response is not only localized in the brain parenchyma but is also a generalized response in the entire organism. The increasing evidence of the presence of this particular inflammatory response in Parkinsonism indicates the need for increased effort to better understand these processes and how they are related to dopaminergic neurodegeneration in an attempt to find a therapeutic strategy capable of stopping or at least slowing down neuronal loss.

In conclusion, many evidences lead to the idea that inflammatory factors may play an important role in Parkinsonian neurodegeneration. However, its function remains unclear. Glial reaction occurring in the SNpc in the areas of dopaminergic neurodegeneration may represent a protective response to neuronal degeneration but at the same time, the uncontrolled release of cytokines and other inflammatory compounds could provoke a harmful effect to the surrounding parenchyma perpetuating the neuronal loss. Inflammatory factors seem to be altered in patients but it is unclear whether antiinflammatory drugs have real beneficial effects in the progression of the disease. In recent years of research, a big progress has been made in the understanding of how local inflammation is related to dopaminergic cell loss and how the control of these factors could have beneficial effects in experimental Parkinsonism. However, the complexity of the disease and the numerous uncontrolled variables found in each patient still make it very difficult to translate the basic experimental conclusions into clinical therapy.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 20

Increase of Secondary Processes of Microglial and Astroglial Cells After MPTP-Induced Degeneration in Substantia Nigra *Pars Compacta* of Non Human Primates

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Abstract Nigral dopaminergic areas from Parkinsonian patients show an increase of reactive astrocytes and active microglia. The reaction of these two cell types is a clear evidence of inflammatory response associated with dopaminergic cell loss. However, the function of this glial reaction remains unclear. This histological hallmark is also reproduced in induced Parkinsonian animals such as MPTP-treated monkeys. In this work, we analyze with confocal microscopy the number of processes of microglial cells and astrocytes in the SNpc of MPTP-treated monkeys and compare with control animals. We observe that secondary branches from microglia and astrocytes increase in MPTP-treated animals, while the scaffold of primary branches does not change. These results demonstrate that glial reaction in MPTP-treated monkeys is characterized by the emission of new filaments after the dopaminergic degeneration, suggesting that glial cells may increase their scanning progress and modify their microanatomy after dopaminergic injury.

Keywords Astroglia • Glial reaction • Microglia • Neuroinflammation • Parkinson's disease

Abbreviations

PD	Parkinson's disease
DAB	Diamino benzidine.
GFAP	Glial fibrillary acidic protein
HLA-DR	Human leukocyte antigen type DR
Iba-1	Ionized calcium-binding adaptor molecule 1
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
SNpc	Substantia Nigra pars compacta
TH	Tyrosine hydroxylase

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Introduction

Inflammatory factors are associated with dopaminergic degeneration in Parkinson's disease (PD). Parkinsonian patients show an increase of the levels of proinflammatory cytokines in blood serum and cerebrospinal fluid (Mogi et al. 1994, 1996; Stypula et al. 1996) and recent evidence, obtained from epidemiological studies, also suggested that the use of antiinflammatory drugs may protect from dopaminergic degeneration (Tansey et al. 2007; Wahner et al. 2007). This idea has also been supported by the evidence that antiinflammatory drugs protect from experimentally induced dopaminergic degeneration in animal models of PD.

Postmortem studies of brains from patients who have died with PD have revealed that inflammatory response in the brain parenchyma is characterized by astroglial (Forno et al. 1992) and microglial activation (McGeer et al. 1988). An increase of reactive astrocytes and activated microglia is observed in the substantia nigra *pars compacta* (SNpc) of PD patients associated with the areas of neuronal loss. However, the function of this glial reaction on dopaminergic cell loss remains poorly understood.

In experimental models of PD, similar reaction is observed. Animals show an increase of glial fibrillary acidic protein (GFAP) positive cells and activated microglial cells in the SNpc due to the induced dopaminergic degeneration. Results from our group demonstrated that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys show an increase of astrocytes immunoreactivity and microglial activation in areas of the SNpc where the neuronal degeneration is higher (Barcia et al. 2004).

It is well known that after injury, astrocytes seem to respond, increasing the intracellular amount of GFAP, but it is not well defined whether astrocytes move to the site of degeneration or change their morphology. In the same way, the activation of microglial cells also occurs after injury but it is not well known whether this activation implies the

increase of different markers, cell division, or infiltration of macrophages from the blood stream.

Recent experiments performed *in vivo* with two-photon microscopy have shown that astrocytes and microglia react in the brain parenchyma after injury (Nimmerjahn et al. 2005; Kim and Dustin 2006). Microglial cells and astrocytes are able to scan constantly the surrounding tissue in normal conditions and rapidly move their processes through the injured areas (Hirrlinger et al. 2004; Nimmerjahn et al. 2005; Kim and Dustin 2006). Astrocytes seem to react after microglial cells but both are able to modify and polarize their processes to the damaged area (Kim and Dustin 2006).

In this work we analyze the processes of astrocytes and microglia in the SNpc of monkeys treated with MPTP to elucidate whether glial cells undergo morphological changes after dopaminergic cell loss.

Material and Methods

Characterization of Monkeys

Twelve adult cynomolgus monkeys (*Macaca fascicularis*) were used for this study. All studies were carried out in accordance with the Guidelines of the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes of the Council of Europe of 2006, the Helsinki Declaration, the International Primatological Society Guidelines, and with the Guide for the Care and Use of Laboratory Animals. Eight of the monkeys were treated by intravenous injections of MPTP according to previous protocols (Herrero et al. 1993). Injections were administered according to each individual's Parkinsonian syndrome to reach a slight/moderate stable and persistent Parkinsonism. The other four animals were used as controls. Motor symptoms were assessed using a previously described rating scale ranging from 0 to 25 (Herrero et al. 1993). The degree of disability increased with every new injection, and although it subsequently fell, it increased again with each dose until it remained stable over several months (1 or 2 years). Susceptible MPTP-treated monkeys showed from moderate (motor score <15) to severe Parkinsonian symptoms (motor score >15) and displayed bradykinesia, akinesia, freezing phenomena, action tremor, paradoxical kinesia, and vertical and horizontal saccadic ocular movements.

Immunohistochemical Procedures

The 50 μ m coronal brain sections were cut serially through the entire brain, and DAB detection or immunofluorescence

was performed as described previously (Thomas et al. 2000; Barcia et al. 2006, 2007), using the following primary antibodies to recognize tyrosine hydroxylase (TH) (1:500, Sheep, Chemicon), GFAP (1:500, Rabbit, Chemicon), HLA-DR (1:50, Mouse DAKO), and Iba-1 (1:500, Rabbit, Wako).

For DAB staining, endogenous peroxidase activity was quenched with 0.3% H_2O_2 in PBS, nonspecific Fc-binding sites were blocked with 10% horse serum and sections were incubated for 48 h at room temperature with primary antibody diluted in PBS containing 1% horse serum and 0.5% Triton-X-100 (antibody solution). Sections were then incubated for 4 h with appropriate biotin-conjugated secondary antibodies (DAKO, Cambridge, UK). Antibody binding was detected using the avidin-biotin peroxidase with diaminobenzidine as chromogen. These sections were mounted on gelatinized glass slides and dehydrated before coverslipping.

For immunofluorescence, a series of sections of each brain were pretreated with 10 mM citrate buffer, at pH 6, for 30 min at 65°C to increase antigen retrieval and penetration of the antibodies into the tissues. Sections were blocked with 1% Triton X-100 for 5 min and 3% normal horse serum in 0.1 M PBS, pH 7.4, for 60 min. Sections were incubated at room temperature for 48 h with combined primary antibodies. For multiple staining, incubation with primary antibodies was followed by 4 h of incubation with the appropriate secondary antibodies, Alexa 488 or Alexa 594 (1:1,000; Molecular Probes).

After washing, the sections were mounted and examined by conventional fluorescence microscopy (Axiolab, Zeiss) and analyzed with confocal microscope (DMIRE2, Leica Microsystems, Exton, PA).

Stereological Quantification

The number of cells in the SNpc was estimated by stereological methods using the optical fractionator probe, as described previously (Barcia et al. 2004). Positive cells were quantified using unbiased stereological methods (Sterio 1984) with a computer-assisted image analysis system (ScionImage) and a Zeiss microscope connected to a digital camera (CoolSnap) through a Zeiss zoom set at 12.5 X and a 0.1 X adapter. The number of cells was measured in 250 μ m sided squares (dissectors), 750 μ m (x) and 750 μ m (y) apart, systematically covering the whole surface area of the analyzed regions. Approximately 25 fields were quantified per section. Using the principle of the optical disector, positive cells were counted only when they cut the top and left hand border of the square as was previously described (Sterio 1984). Four representative sections were quantified for each animal at four levels of the SNpc from rostral to caudal

levels. Results are expressed as an estimation of the absolute number of positive cells in the regions analyzed.

Confocal Analysis

The primary and secondary ramifications of GFAP and Iba-1⁺ cells was counted in 15 cells per animal using the Leica confocal software. The quantification was done by a researcher who did not know the treatment of each animal (blind study). The brain sections were examined using a Leica DMIRE2 confocal microscope with the 63x oil objective and Leica Confocal Software (Leica Microsystems Heidelberg 19 GmbH). Each cell was scanned determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting [For further details of confocal imaging see previous publications (Barcia et al. 2006, 2008)]. Images containing positive cells were randomly captured in the SNpc in both groups of animals. The number of GFAP or Iba-1⁺ branches of each cell was quantified using confocal microscope software.

Statistical Analysis

Viability data were expressed as mean $\pm$ SEM and evaluated by the Mann and Whitney test. Differences were considered significant if $p < 0.05$.

Results

To demonstrate the dopaminergic cell loss induced by MPTP, sections from rostral to caudal levels of the SNpc were immunostained for TH. Dopaminergic neurons were clearly stained and quantification was performed by stereology. Monkeys treated with MPTP showed a high degree of neuronal loss in the SNpc that was higher in rostral sections than the caudal sections (Fig. 1). Adjacent sections were immunostained for activated microglia (expressing HLA-DR). HLA-DR⁺ cells were observed in the SNpc and quantified. Quantification showed an increase of activated microglia in the SNpc of MPTP-treated monkeys, which was higher in rostral sections (Fig. 1). Adjacent sections were also stained for GFAP to analyze reactive astrocytes. An increase of GFAP⁺ cells was also observed in the SNpc of MPTP-treated monkeys, which was higher in rostral and intermediate sections and decreased in caudal sections (Fig. 1).

Sections from the rostral part of the SNpc were stained with immunofluorescence for Iba-1 or GFAP to analyze in

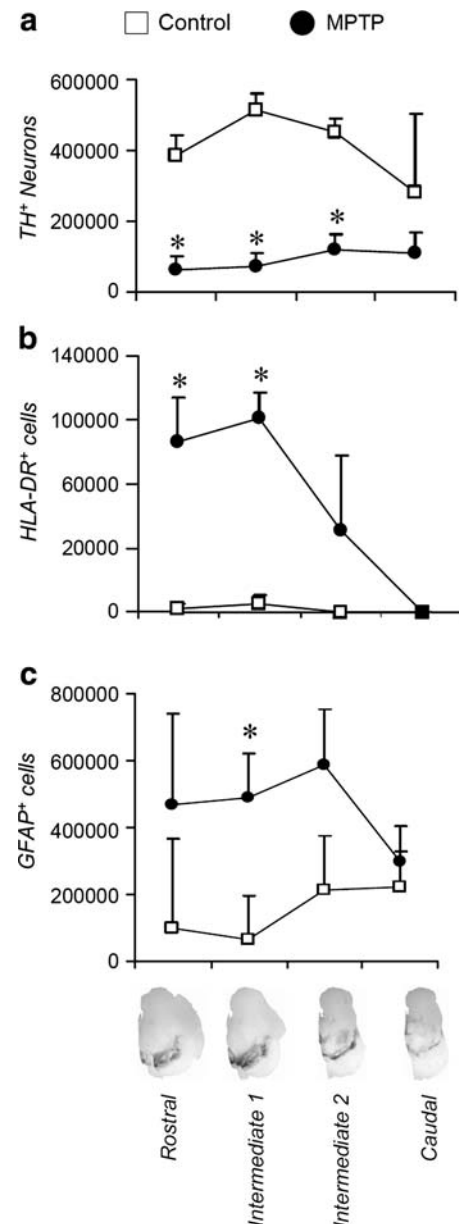


Fig. 1 Stereological quantification of the number of dopaminergic neurons (TH⁺), activated microglia (HLA-DR⁺) and reactive astrocytes (GFAP⁺) in the SNpc of Parkinsonian monkeys. Each graph represents the stereological estimation of the number of cells from rostral to caudal levels of the SNpc (Rostral, Intermediate 1, Intermediate 2 and Caudal). A shows the quantification of dopaminergic neurons. The higher neuronal loss is observed in rostral and intermediate sections but not in caudal sections. Panel B illustrates the quantification of activated microglial cells. The number of activated microglial cells was higher in rostral levels than caudal. Quantification of the number of reactive astrocytes (GFAP⁺) is shown in panel C. The higher increase of reactive astrocytes is observed at rostral and intermediate sections but not in caudal ones. A representative section of each area is illustrated at the bottom of the graph

detail the glial cells with a confocal microscope and quantify the number of primary and secondary branches of microglia and astrocytes in the SNpc. Immunofluorescence with Iba-1

revealed typical microglial cells in the SNpc clearly marking the cell body and processes. The quantification of the number of the primary branches showed that there were no differences between Iba-1⁺ cells from control animals and MPTP-treated animals (Fig. 2). However, the quantification of the secondary branches showed a significant increase in the animals treated with MPTP (Fig. 2). On the other hand,

immunofluorescence for GFAP revealed typical astrocytes in the SNpc marking also the cell body and processes. The quantification of the number of the primary branches showed no differences between GFAP⁺ cells from control animals and MPTP-treated animals. However, the quantification of secondary branches also revealed a significant increase in the SNpc of MPTP-treated monkeys (Fig. 2).

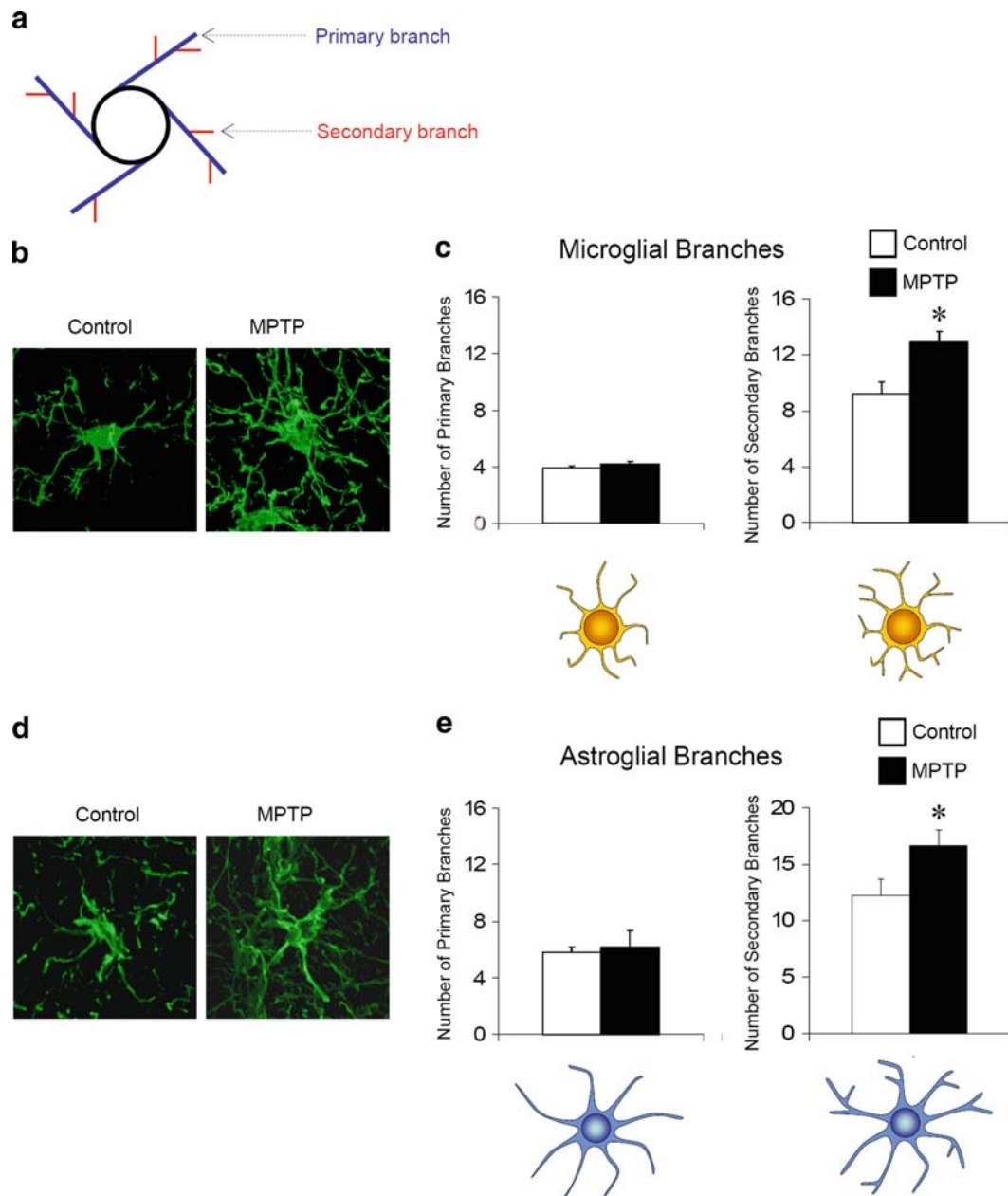


Fig. 2 Secondary branches of astrocytes and microglia are increased in SNpc after MPTP exposure. Panel A shows a schematic representation of a cell body with the criteria of quantification of primary and secondary branches. Panel B shows confocal pictures of a characteristic microglial cell, immunostained for Iba-1, from the SNpc of a control monkey and a MPTP-treated monkey. In Panel C is shown the quantification of primary and secondary branches of microglial cells from the SNpc of control and MPTP-treated monkeys. An increase of secondary branches was found in MPTP-treated monkeys. * $p < 0.05$. Panel D shows two confocal pictures illustrating the characteristic shape of a GFAP⁺ astrocyte in the SNpc of a control and MPTP-treated monkey. Panel E illustrates the quantification of primary and secondary branches of astrocytes of the SNpc. A statistically significant increase of secondary branches was observed in the astrocytes of the SNpc of Parkinsonian monkeys. * $p < 0.05$

Discussion

In this work, we observed an increase of microglia and astrocytes in the SNpc associated with the areas of dopaminergic neurodegeneration principally observed in the rostral areas. We previously described that the neurodegeneration in MPTP-intoxicated monkeys is located in the ventrolateral area of the SNpc (Barcia et al. 2004). However, in this work, we analyze from rostral to caudal areas of the SNpc, observing that rostral sections show a higher degree of dopaminergic degeneration than caudal (Fig. 1). This result correlates with the increase of activated microglia and reactive astrocytes that is higher at the levels of the SNpc where the dopaminergic degeneration is higher. These data confirm that inflammatory reaction accompanies neuronal degeneration and occurs in the surrounding areas of dopaminergic cell death. Nevertheless, it remains unknown whether this inflammatory reaction exerts a protective effect or could have harmful consequences in the surrounding tissue.

Detailed analysis of microglial and astroglial cells from sections from rostral areas of the SNpc revealed that this glial reaction involves morphological changes of the cells. The quantification of our confocal images shows that secondary branches of astrocytes and also microglia appear increased after MPTP exposure while primary branches remain unchanged.

Studies performed with two-photon microscopy have shown that microglial cells are able to constantly emit processes (secondary or tertiary branches) to scan the surrounding area but they maintained an unchanging scaffold (primary branches and cell body) that scarcely moves. This processes formation increases in microglia following laser microlesions or LPS inoculation performed in the brain parenchyma (Nimmerjahn et al. 2005). In this study, we demonstrate that after dopaminergic cell death in the SNpc, microglial cells show an alteration of their shape characterized by the increase of secondary branches maintaining an unaltered scaffold of primary branches. This result suggests that MPTP lesion induces morphological changes in microglial cells probably due to the neuronal loss occurring in the vicinity or due to the toxicity and inflammation caused in the surrounding parenchyma.

Other studies also using two-photon microscopy have revealed that astrocytes also respond to injuries in the brain parenchyma (Kim and Dustin 2006). Similar to the results observed in microglia, astrocyte processes also show spontaneous motility and extension/retraction cycles (Hirrlinger et al. 2004) and react after injury. Accordingly, in our results we observed an increase of secondary processes in astrocytes of the SNpc after MPTP-induced neurodegeneration. Interestingly, as microglial cells, primary branches do not change after MPTP insult, which suggests that the astrocytic and microglial reaction has a similar pattern. However,

previous reports have demonstrated that this glial reaction and their morphological changes occur with a clear timing. Microglial cells seem to respond first to the injured area, while astrocytes appear to be slower in displaying this type of morphological change (Nimmerjahn et al. 2005; Kim and Dustin 2006).

These results demonstrate that glial cells respond with morphological changes in the SNpc after induced dopaminergic cell loss. This response is suggested as a part of the inflammatory response observed in the SNpc in Parkinsonism and could contribute to the motility of glial cells to the injured parenchyma. This emission of a higher number of processes may play an important role on the protective or harmful effect of glial cells on remaining neurons. These morphological changes are probably needed to increase the scanning area of the damaged parenchyma or increase the release of different growing factors or cytokines. However, more experiments are needed to unravel the mechanisms of glial inflammatory reaction after dopaminergic cell death in Parkinsonism and how this response may affect neurodegeneration.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 21

Distinct Effects of Intranigral L-DOPA Infusion in the MPTP Rat Model of Parkinson's Disease

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Abstract The potential neuroprotective or neurotoxic effects of 3,4-dihydroxyphenylalanine (L-DOPA) are yet to be understood. We examined the behavioral, immunohistochemical, tyrosine hydroxylase (TH) expression and neurochemical parameters after an intranigral administration of L-DOPA (10 μ M) in rats. L-DOPA elicited a 30.5% reduction in dopaminergic neurons, while 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (100 μ g μ L⁻¹) produced a 53.6% reduction. A combined infusion of MPTP and L-DOPA generated a 42% reduction of nigral neurons. Motor parameters revealed that both the MPTP and L-DOPA groups presented impairments; however, the concomitant administration evoked a partial restorative effect. In addition, MPTP and L-DOPA separately induced reductions of TH protein expression within the substantia nigra. In contrast, the coadministration of MPTP and L-DOPA did not demonstrate such difference. The striatal levels of dopamine were reduced after MPTP or L-DOPA, with an increased turnover only for the MPTP group. In view of such results, it seems reasonable to suggest that L-DOPA could potentially produce dopaminergic neurotoxicity.

Keywords Animal model • L-DOPA • MPTP • Neurotoxicity • Parkinson's disease • Tyrosine hydroxylase

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Abbreviations

DA	Dopamine
L-DOPA	3,4-dihydroxyphenylalanine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TH-ir	Tyrosine hydroxylase immunoreactive

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder the classical standard pharmacological protocol of which is polytherapy. The multitude of clinical approaches is detrimental to the overall comprehension of the physiological and molecular mechanisms that are affected by each drug individually. The possible neurotoxic consequence entailed by a given drug becomes difficult to trace as these effects are almost indistinguishable in clinical trials. Dopamine (DA) replacement by the administration of 3,4-dihydroxyphenylalanine (L-DOPA) is a gold standard drug for the symptomatic treatment of PD (Tedroff 1997). This therapy dramatically improves Parkinsonian symptoms. L-DOPA is converted by neuronal aromatic L-amino acid decarboxylase into DA, hence restoring DA levels in surviving neurons, but not halting neuronal death (Basma et al. 1995). Nevertheless, it is well known that chronic use of L-DOPA, especially in patients in advanced stages of the disease, leads to the development of motor complications that are very resistant to therapy, which aggravates disability in PD patients (Benbir et al. 2006). Yet another characteristic of L-DOPA administration is the occurrence of on-off motor variations, which is recognized by involuntary dyskinesia and psychiatric complications.

Animal models of PD have reported that acute L-DOPA treatment reversed the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced decrease in locomotion, rearing,

and total activity (Sundstrom et al. 1990). For instance, 35 days of intraperitoneal L-DOPA treatment resulted in significant dyskinesia (Lundblad et al. 2004). Preclinical outcomes of in vitro models of neurodegeneration describe the neurotoxic effects of L-DOPA (Alexander et al. 1997; Ziv et al. 1997; Mytilineou et al. 2003), whereas in vivo trials in animal models provided controversial results (Muller et al. 2004). The variety of L-DOPA effects, in terms of its neurotoxic or neuroprotective potential and the mechanism through which DA is generated, exerts an effect despite the absence or reduction of DA innervation to target sites in the basal ganglia (Kostrzewa et al. 2005).

In this study, we hypothesized that an intranigral administration of L-DOPA 10 μ M, which is a particular concentration that undergoes an important biotransformation within the substantia nigra (SN) (Sarre et al. 1998), could result in dopaminergic neurotoxicity, supporting the idea of a dual role of L-DOPA, and therefore, that of DA, in the nigrostriatal pathway. The aim of this study was to examine the effects of a single intranigral administration of L-DOPA, using as comparative parameter the experimental model of PD induced by the intranigral administration of MPTP in rats, which recapitulates several features of the disease (Lima et al. 2006, 2007; Reksidler et al. 2007, 2008). To obtain the needed data we verified the extent of dopaminergic neuronal loss inflicted by MPTP, L-DOPA, and their combination, named the MPTP+L-DOPA group, by estimating the number of tyrosine hydroxylase immunoreactive (TH-ir) neurons within the substantia nigra pars compacta (SNpc). Moreover, neurochemical analyses of the striatal content of DA and metabolites were conducted. Additionally, motor system function was examined under the open-field test. Complementarily, western blotting analyses were performed to verify the TH protein expression along the nigrostriatal pathway.

Material and Methods

Animals

Male Wistar rats (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia-CEDEME facility of Universidade Federal de São Paulo-Escola Paulista de Medicina) weighing 280–320 g at the beginning of the experiments were used. Fifty rats were used in the behavioral tests, and the same animals were used for the immunohistochemistry analyses. An additional set of 45 rats was used for neurochemical and western blotting experiments. After the surgical procedure, the animals were returned to the same home cages where they were housed and maintained in a

temperature-controlled room, located in our facility, ($22 \pm 2^\circ\text{C}$) on a 12 h light-dark cycle (lights on 7:00 a.m.) with free access to food and water. The animals used in this study were maintained and handled in accordance with the guidelines of the Ethical Committee of Universidade Federal do Paraná (UFPR) and Universidade Federal de São Paulo (UNIFESP), Brasil.

Experimental Protocol

On the first day of the experiment, the animals received bilateral guide cannula implants allowing for the introduction of an injection needle into the medial SNpc. After 7 days of recovery from the stereotaxic surgery, intranigral infusions of MPTP or L-DOPA were performed and another group received MPTP followed by L-DOPA in the same animals. Twenty-four hours after their infusion procedures, all the groups had their motor behavior recorded in the open-field. After assessment of their motor behavior, all the animals were intracardially perfused for brain fixation allowing for histological exam to determine the neuronal loss in the SNpc. Another set of animals was operated and underwent the same infusion procedure, but this set of animals was used for striatal neurochemical exam and TH protein expression study in the SN and striatum tissues.

Stereotaxic Surgery and Intranigral Injections

The animals were distributed at random, equally into the following groups: control ($n=19$), sham ($n=19$), MPTP ($n=19$), L-DOPA ($n=19$), and MPTP+L-DOPA ($n=19$). Rats were anesthetized with diazepam (10 mg kg^{-1} i.p.) ketamine (90 mg kg^{-1} i.p.). All the groups, with the exception of the control, received a bilateral guide, cannula implanted 2.0 mm above the SNpc according to the following coordinates: anteroposterior (AP): -5.0 mm from bregma; mediolateral (ML): ± 2.1 mm from midline; and dorsoventral (DV): -7.8 mm from skull, adapted from Paxinos and Watson (2005). All the operated rats received penicillin G-procaine (20,000 U in 0.1 ml, i.m.) after surgery.

After 7 days, the animals were manipulated and immobilized to perform the drug injections. A 30-gauge stainless injection needle was bilaterally introduced through the cannula for the administration of 1 μ L of MPTP (100 $\mu\text{g}\mu\text{L}^{-1}$, prepared in sterile saline 0.9%, Sigma), 1 μ L of L-DOPA (10 μM , prepared in sterile saline 0.9%, Sigma), which is a concentration ten fold greater than the cerebrospinal concentration of L-DOPA in human PD patients (Olanow et al. 1991), and 1 μ L of MPTP (100 $\mu\text{g}\mu\text{L}^{-1}$) + 1 μ L of L-DOPA

(10 μ M). The control of the flow of the injection was made by using an electronic pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0.40 μ L min⁻¹ for 2.5 min, followed by 2 min with the needle in the injection site to avoid reflux. Sham operations followed the same procedure but were administered 1 μ L of sterile saline 0.9%.

Behavioral Assessment

To determine behavioral alterations induced by intranigral administration of MPTP, L-DOPA, and their combination, we resorted to the open-field as a test of general activity. Fifty rats were distributed into five groups: nonoperated (n = 10), sham (n = 10), MPTP (n = 10), L-DOPA (n = 10), and MPTP+L-DOPA (n = 10). Each group, except the control and sham groups, received a bilateral administration of MPTP, L-DOPA, or both. The open-field was performed 24 h after the intranigral administrations to assess the maximal motor deficit elicited by MPTP (Lima et al. 2006).

We employed the open-field constructed according to Broadhurst (1960). The testing arena was round with a diameter of 97 cm. The circular wall was 32.5 cm high and was constructed of aluminum sheeting as was the arena floor. The walls were painted white. The arena floor was divided into three concentric circles. The inner circle had a diameter of 23 cm; the second circle had a diameter of 61 cm and the arena wall defined the outside circle. Each circle was divided into equal sized areas. The number of areas in the inner, middle, and outer circles was 1, 6, and 12 respectively. A 100-W ceiling light was situated 48 cm above the arena floor. Rats were observed individually for 5 min and the different groups were inter-mixed. Hand-operated counters were used to score the following parameters: latency time (time taken to initiate movement), locomotion frequency (number of floor units entered), rearing frequency (number of times the animals stood on their hind legs), and immobility time (number of seconds of lack of movement during testing). The apparatus was washed with a water-ethanol (5%) solution before behavioral testing to eliminate possible bias due to odors left by previous rats.

TH Immunohistochemistry

For the immunohistochemical study, the rats were deeply anesthetized with ketamine immediately after the behavioral test and were intracardially perfused with saline first, then with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and were immersed for 1 week in that fixative solution

at 4°C. Subsequently, the brains were placed in 30% sucrose solution for 48 h before sectioning. Four series of 30 μ m thick sections were cut on a cryostat in the frontal plane and collected from the caudal diencephalon to the caudal midbrain. Tissue sections were incubated with primary antibody anti-TH, raised in rabbits, diluted in PBS containing 0.3% Triton X-100 (1:500; cat # AB152 Chemicon, CA, USA) overnight at 4°C. Biotin-conjugated secondary antibody incubation (1:200 cat # S-1000 Vector Laboratories, USA), was allowed for 2 h at room temperature. After several washes in PBS, antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat # PK6101, Vector Laboratories, USA) followed by 3,3'-diaminobenzidine reaction with nickel enhancement. Slides were then dehydrated in ascending ethanol concentrations, cleared in xylene and coverslipped. An adjacent series was stained with cresyl violet to serve as a reference series for cytoarchitectural purposes.

To estimate the extent of neuronal loss within the mid-brain due to MPTP, L-DOPA, and MPTP+L-DOPA, stereological methods were adopted. An operator performed blind counts, first assessing the dopaminergic cell population within the SNpc in control and sham animals. These were found to have preserved a normal cytoarchitectural appearance and TH immunostaining. Subsequently, the same investigator performed yet another blind count, assessing the same cell population from MPTP, L-DOPA, and MPTP+L-DOPA groups. All cell counts were done making use of the software Image-Pro Express 6. The selected areas were digitized through a digital camera DP71 (Olympus Optical Co, Japan) using an Olympus microscope BX50.

Neurochemical Determination of DA and Metabolites

Forty five rats were distributed into five groups: nonoperated (n=6), sham (n=6), MPTP (n=6), L-DOPA (n=6), and MPTP+L-DOPA (n=6). The endogenous levels of DA and its nonconjugated metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were assayed by reverse-phase HPLC with electrochemical detection. Briefly, the system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150 $\times$ 4.6 mm i.d., 4 μ m particle size) fitted with a 4.0 $\times$ 3.0 mm precolumn (Security Guard Cartridges Fusion-RP). An electrochemical detector (ESA Coulochem Electrochemical Detector) equipped with a guard cell (ESA 5020) with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); a LC-20AT pump (Shimadzu) equipped with a manual Rheodyne 7725 injector with a 20 μ L loop. Oxidizing potentials were set

at 100 mV for the first electrode and at 450 mV for the second electrode. DA and metabolites were detected at the second electrode.

Each striatum was weighed in a measured volume (20% wt vol⁻¹) of 0.1 M perchloric acid and sodium metabisulfite 0.02% containing the internal standard 3,4-dihydroxybenzylamine at 100 ng mL⁻¹. The tissue samples were homogenized with an ultrasonic cell disrupter (Sonics). After centrifugation at 10,000 g for 30 min at 4°C, 20 µL of the supernatant was injected into the chromatograph. The mobile phase, used at a flow rate of 1 mL min⁻¹, had the following composition: 20 g citric acid monohydrated (Merck), 200 mg octane-1-sulfonic acid sodium salt (Merck), 40 mg ethylenediaminetetraacetic acid (EDTA) (Sigma), 900 mL HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a Nylon microfilter (pore size, 0.45 µm; Bioanalytical Systems, West Lafayette, IN). Methanol was added to give a final concentration of 10% (v/v). The concentrations of DA, DOPAC, and HVA were calculated using standard curves that were generated by determining in triplicate the ratios between three different known amounts of the internal standard. The unit was expressed as ng/g wet weight.

TH Protein Expression

To determine the expression profile of TH, we used 15 rats distributed into equivalent five groups mentioned previously: control (n=3), sham (n=3), MPTP (n=3), L-DOPA (n=3), and MPTP+L-DOPA (n=3). Animals were killed by decapitation and their brains were quickly dissected. The SN and striatum were immediately frozen in dry ice and stored at -80°C until the lysis procedure was performed in a 1.5 mL Eppendorf tube by sonication in presence of an ice-cold buffer containing 50 mM Tris (pH 8.0), 250 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 2 mM EDTA, 1 mM dithiothreitol (DTT), 20 µM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (Complete tablet; Roche, Indianapolis, IN, USA).

After incubation on ice for 30 min, extracts were centrifuged at 12,000 × g for 40 min at 4°C, and the supernatants for protein extracts were collected and stored at -80°C for further western blotting analysis. The aliquot of supernatant was collected for total protein analysis (Lowry et al. 1951). Samples containing equal amounts of total protein (5 µg per lane) were boiled with SDS sample buffer and electrophoresed in 10% SDS-polyacrylamide gels in Mini Protean II Dual Slab Cell (Bio-Rad, USA). Proteins were electrophoretically transferred to PVDF membranes using a Mini transblot electrophoretic transfer cell (Bio-Rad, USA).

Each membrane was blocked for 1 h in 10% nonfat dry milk/0.5% Tween-20 in Tris-buffered saline. Subsequently, each PVDF membrane was probed overnight at 4°C with mouse monoclonal antibodies against TH (1:5,000; cat # T2928 Sigma, USA) or β-tubulin (1:500; cat # MAB1637 Chemicon, USA) followed by several washes in TBST and incubation with an adequate secondary horseradish peroxidase-conjugated antibody (1:5,000; cat # 30021019 GE, USA) for 60 min, and visualized by chemiluminescence (cat # sc 2048 Santa Cruz Biotechnology, USA). The bands were quantified by using the software ImageJ 1.32j public domain (<http://rsb.info.nih.gov/ij/>).

Statistical Analysis

Differences in the number of cell counts and Western blotting data underwent analysis of variance (ANOVA) followed by Newman-Keuls test. The open-field data were concluded to be parametric by the Bartlett's test. ANOVA with repeated measurements was employed to detect differences among the treatments. For the behavioral and neurochemical data, the Tukey test was used as post hoc when indicated by ANOVA. Differences were considered significant if $P < 0.05$. The values were expressed as mean ± S.E.M.

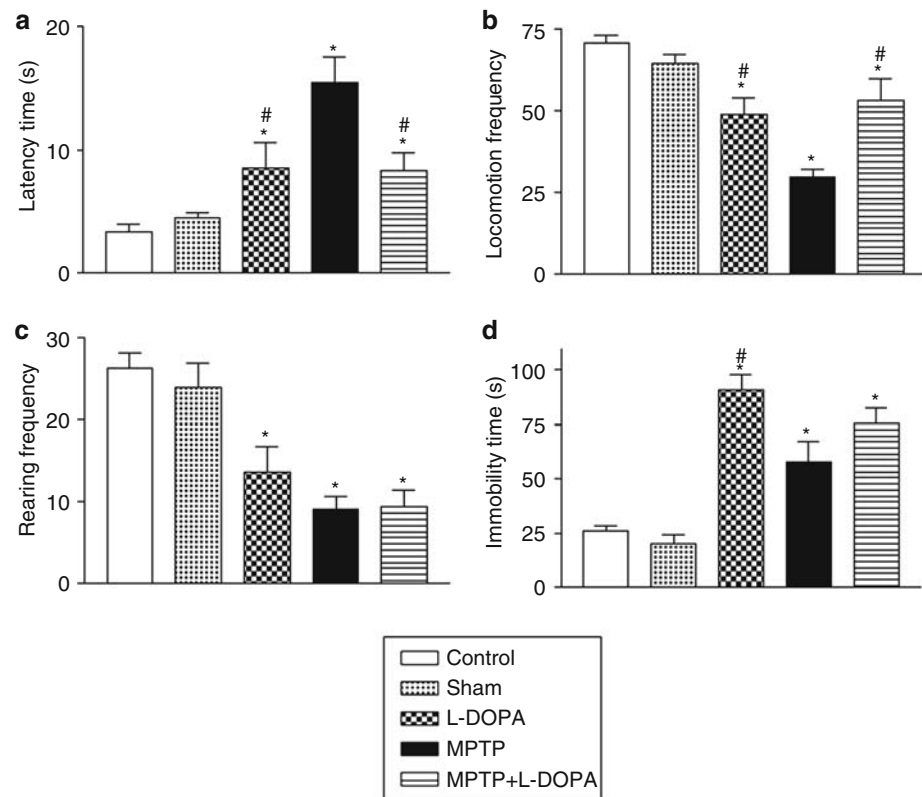
Results

Behavioral Assessment

The data obtained for latency in initiating movement (Fig. 1a) revealed a significant increase in this parameter in the groups administered with L-DOPA ($P < 0.05$), MPTP ($P < 0.001$), and MPTP+L-DOPA ($P < 0.05$) in comparison with the control group [$F(4,32) = 14.57$; $P < 0.05$]. The MPTP group exhibited a more pronounced increase in this parameter in comparison with the L-DOPA ($P < 0.05$) and MPTP+L-DOPA ($P < 0.01$) groups. Indeed, the immobility time presented similar alterations.

The analyses of ambulatory behavior were determined by the locomotion frequency depicted in Fig. 1b. That figure shows a significant reduction in the groups that received L-DOPA ($P < 0.05$), MPTP ($P < 0.001$), and their combination MPTP+L-DOPA ($P < 0.05$) in comparison with the control group [$F(4,32) = 14.19$; $P < 0.05$]. As expected, the animal model of PD induced by the MPTP group presented a significant reduction in the locomotion frequency in comparison with the L-DOPA ($P < 0.05$) and MPTP+L-DOPA ($P < 0.01$) groups. Rearing frequencies (Fig. 1c) indicated

Fig. 1 Effect of intranigral infusions of MPTP, L-DOPA or MPTP+L-DOPA in the open-field test. (a) Latency time, (b) locomotion frequency, (c) rearing frequency, (d) immobility time. The values are expressed as mean $\pm$ S.E.M. ($n=10$ animals per group). * $P<0.05$ compared to control group; # $P<0.05$ compared to MPTP group. ANOVA followed by the Tukey test



that L-DOPA ($P<0.01$), MPTP ($P<0.001$), and MPTP+L-DOPA ($P<0.001$) showed a significant decrease in this exploratory parameter when the animals were compared with the control group [$F(4,32)=11.64$; $P<0.01$].

Figure 1d depicts a significant increase in this parameter in the following groups: L-DOPA ($P<0.001$), MPTP ($P<0.01$), and MPTP+L-DOPA ($P<0.001$) in comparison with the control [$F(4,32)=31.79$; $P<0.01$]. Moreover, the L-DOPA group presented a significant increase in this motor sign when compared with the MPTP ($P<0.01$) group.

TH Immunohistochemistry

The effects of L-DOPA, MPTP, and MPTP+L-DOPA on the number of TH-ir neurons were examined by immunohistochemistry. As depicted in Figs. 2 and 3, the intranigral administration of MPTP, L-DOPA, and MPTP+L-DOPA caused a similar loss of TH-ir neurons within the SNpc [$F(3,30)=55.93$; $P<0.001$] in comparison with the control group. MPTP administration provoked the more pronounced loss (53.6%) of TH-ir neurons in the SNpc ($P<0.001$) in comparison with the control group. Otherwise, the L-DOPA group presented a 30.5% ($P<0.001$) reduction in the number of TH-ir neurons within the SNpc in comparison with the control group. Conversely, the number of TH-ir neurons

from the L-DOPA group was significantly increased (23.2%; $P<0.001$) in comparison with the MPTP group. The combination of MPTP and L-DOPA also resulted in a significant loss of 42% ($P<0.001$) of the TH-ir neurons within the SNpc, compared with the control group.

Neurochemical Determination of DA and Metabolites

Figure 4a, illustrates that levels of DA were significantly lower in the striatum of L-DOPA ($P<0.001$), MPTP ($P<0.001$), and L-DOPA+MPTP ($P<0.001$) groups in comparison with sham and control groups [$F(4,24)=5.45$; $P<0.001$]. This figure also demonstrates that DOPAC levels were not altered for the groups treated with L-DOPA when compared with control and sham groups (Fig. 4b). However, the MPTP group revealed a significant increase in the DOPAC level in comparison with the control ($P<0.05$), sham ($P<0.05$), and L-DOPA ($P<0.05$) groups [$F(4,28)=3.91$; $P<0.01$] (Fig. 4b). Similarly, HVA showed an increase solely in the striatum of the MPTP group, when compared with the control ($P<0.05$), sham ($P<0.01$), L-DOPA ($P<0.01$), and MPTP+L-DOPA ($P<0.01$) groups [$F(4,32)=5.47$; $P<0.002$] (Fig. 4c).

Fig. 2 Photomicrograph of representative sections of tyrosine hydroxylase immunoreactive neurons (TH-ir) in the substantia nigra of the following groups: (a) Control, (b) Sham, (c) MPTP, (d) L-DOPA, (e) MPTP+L-DOPA. Panel f is a schematic drawing from the area previously depicted (Paxinos and Watson 2005). Legend *cp* cerebral peduncle basal, *SNpc* substantia nigra pars compacta, *SNr* substantia nigra reticulata, *VTA* ventral tegmental area, scale bar 500µm

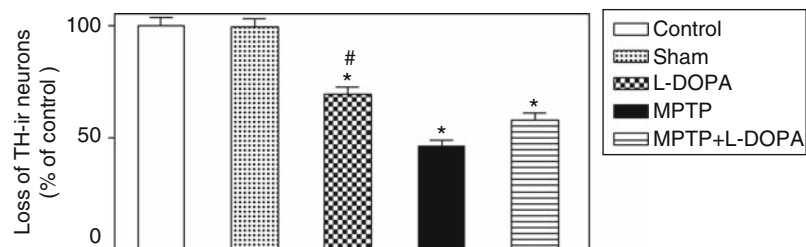
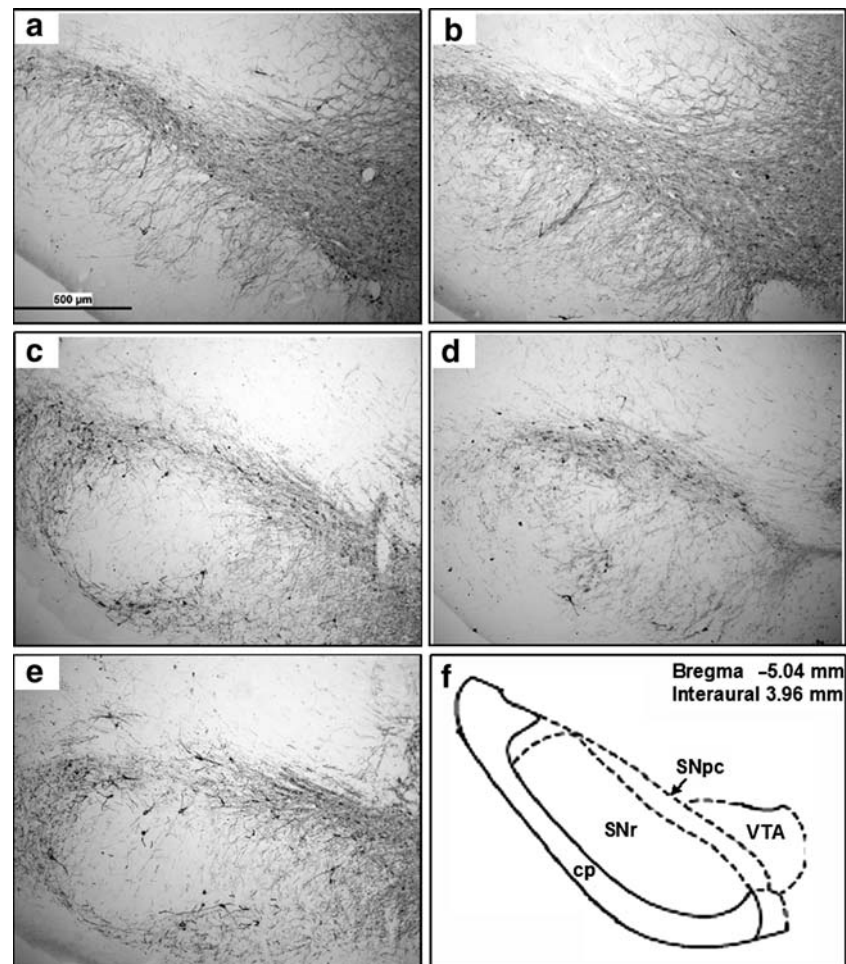


Fig. 3 Effects of intranigral infusions of MPTP, L-DOPA or MPTP+L-DOPA on the percentage of loss of tyrosine hydroxylase immunoreactive neurons (TH-ir) in the substantia nigra pars compacta SNpc of rats. The values are expressed as mean $\pm$ S.E.M. compared to the control group ($n=10$ animals per group). * $P < 0.05$ compared to the control group; # $P < 0.05$ compared to MPTP group; ANOVA followed by the Newman-Keuls test

TH Protein Expression

Western blot analysis of the SN and the striatum revealed a single band of molecular mass of 60 kDa in all groups. Densitometric analyses showed that intranigral administrations of MPTP and L-DOPA produced, in their respective groups, a significant decrease of TH expression in the SN

[$F(4,45)=4.05$; $P < 0.05$] in comparison with the controls (Fig. 5a). The group lesioned with MPTP presented a significant reduction of about 30% ($P < 0.05$) in the TH expression within the SN. Similarly, the L-DOPA group presented a reduction of 28% ($P < 0.05$) in the expression of this protein in the SN. The analysis of the SN obtained after MPTP+L-DOPA administration revealed an absence of

Fig. 4 Neurochemical determination of DA and metabolites in the striatum. Panel (a) depicts the levels of DA, panel (b) levels of DOPAC and panel (c) levels of HVA. The values are expressed as mean $\pm$ S.E.M. ($n=8$ animals per group). * $P<0.05$ compared to control group; # $P<0.05$ compared to MPTP group. ANOVA followed by the Tukey test

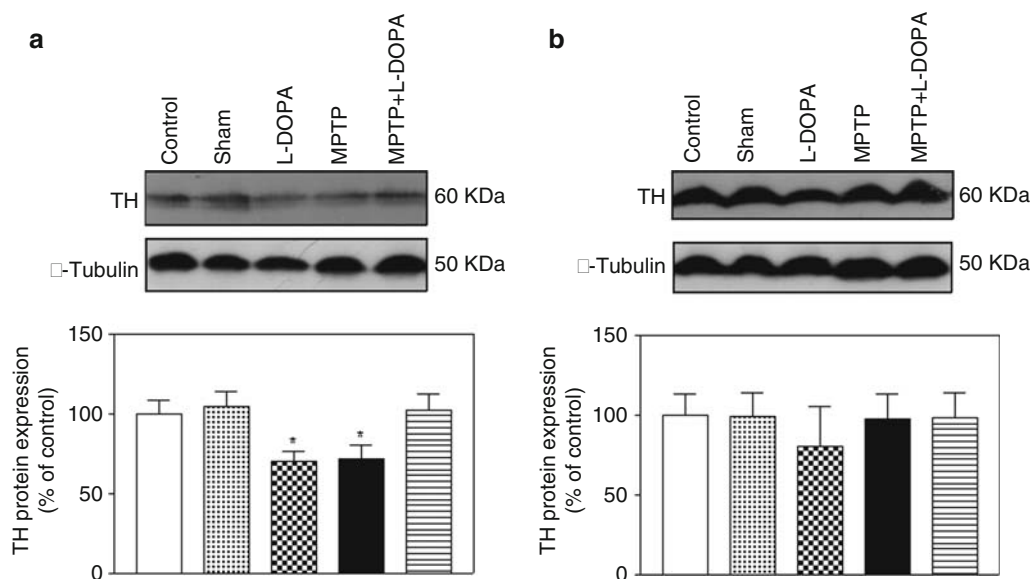
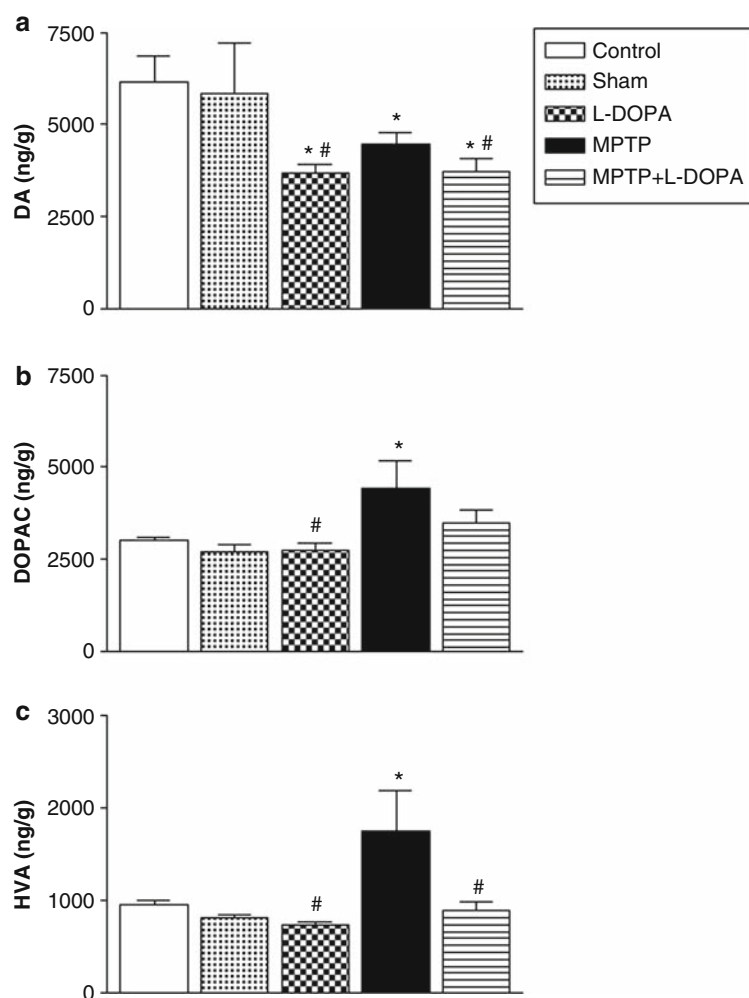


Fig. 5 Western blot analyses of TH expression in the substantia nigra (a) and striatum (b) of groups which received intranigral infusions of MPTP, L-DOPA or MPTP+L-DOPA. A representative immunoblot is shown. The values are expressed as mean $\pm$ S.E.M. for three animals in each group. * $P<0.05$ compared to the control group. ANOVA followed by the Newman-Keuls test

statistical difference in TH expression in comparison with the control group. The analyses of the striatum tissue did not exhibit significant variations in the TH protein expression among the groups included in this study (Fig. 5b).

Discussion

Our experiments demonstrated that a single intranigral administration of L-DOPA induced nigrostriatal dopaminergic destruction similar to that inflicted by MPTP. Analyses of TH protein expression revealed that L-DOPA caused a reduction in the expression of this protein, one very similar to that caused by MPTP in the SN. In parallel, the content of DA was reduced in the striatum after MPTP or L-DOPA, but an increased DA turnover was detected only for the MPTP group. Of note, the combined administration of MPTP and L-DOPA resulted in a relevant reduction of neurotoxicity in TH expression within the SN than that inflicted by MPTP and L-DOPA alone, as demonstrated in our TH expression experiments.

L-DOPA is the most commonly prescribed drug for the symptomatic treatment of PD. Despite this, L-DOPA *per se* presents a serious limitation due to its pharmacological mechanisms, which depend upon the integrity of a minimal neuronal population capable of converting this precursor into DA. Unfortunately, L-DOPA and other anti-Parkinsonian drugs are ineffective in controlling ongoing cell death, and more recently, the notion that L-DOPA can produce dopaminergic neurotoxicity has gained strength (Bendir et al. 2006). Sarre et al. (1998) confirmed that there is an important biotransformation of L-DOPA into DA within the SN of rats infused with L-DOPA directly into this structure. Consequently, it has been postulated that high DA concentrations formed by L-DOPA may cause side effects in PD patients. There are reports stating that approximately 50% of the patients develop motor complications 5 years after the initiation of L-DOPA therapy. This figure increases to approximately 70% after 15 years (Miyawaki et al. 1997). The incidence of such motor problems reaches almost 100% in patients with early onset of PD (Quinn et al. 1987; Golbe 1992; Schrag and Quinn 2000; Thobois et al. 2005).

Our behavioral results partially corroborate these previous data, which denote that L-DOPA alone produced some level of similarity with MPTP, in terms of impairment in the motor parameters. The analysis of latency time revealed that L-DOPA caused a smaller increase in this time, compared with MPTP. This augmented latency time can be related to the manifestation of bradykinesia that occurs in PD. Similarly, the MPTP+L-DOPA group presented approximately

the same profile of latency time as the L-DOPA group. In this sense it is possible to suggest that intranigral L-DOPA treatment, right after MPTP lesion, resulted in distinct modulatory locomotion patterns in the MPTP+L-DOPA and L-DOPA groups. An analogous situation of responses was observed in relation to locomotion frequency parameter. The L-DOPA, MPTP, and MPTP+L-DOPA groups showed significant reductions of locomotion frequencies in comparison with the control group. Although the hypokinesia observed in the two groups treated with L-DOPA is a characteristic feature of a PD model, a significant increase in locomotion frequency in the L-DOPA groups was found in comparison with the MPTP group. Such a result reinforces the notion that patterns of dopaminergic modulation elicited by L-DOPA may exist. Additionally, rearing frequency was reduced in the experimental groups in comparison with controls. Such observations demonstrate the property of MPTP and L-DOPA in the induction of dopaminergic impairment according to the experimental protocol adopted. Immobility time was the only behavioral parameter that L-DOPA alone produced, an effect similar to that of MPTP in the impairment of motor function. The evidence, so far, suggests the possible existence of different patterns of modulatory effects in the dopaminergic nigrostriatal pathway inflicted by L-DOPA.

Several studies have demonstrated that the intranigral administration of MPTP is an established model of the early phase of human PD (Da Cunha et al. 2001, 2002; Miyoshi et al. 2002; Ferro et al. 2005; Braga et al. 2005; Perry et al. 2005; von Bohlen und Halbach 2005; Lima et al. 2006, 2007; Reksidler et al. 2007, 2008). At this point, a note of caution should be added: we evaluated the effects produced by L-DOPA, on dopaminergic neurons, using the MPTP model of PD as a standard of comparison. In this sense, we decided to adopt the same protocol of administration (intranigral) for both substances (MPTP and L-DOPA). Moreover, such approach of neurodegeneration in rats is usually performed with a single intranigral administration of MPTP producing massive effects 24 h after its intranigral infusion (Lima et al. 2006, 2007; Reksidler et al. 2007). Otherwise, a previous study demonstrated that repeated intranigral MPTP administrations did not generate progressive neuronal death, indicating that a single MPTP administration produces a very similar pattern of alterations (Reksidler et al. 2008).

Similar to the MPTP-induced lesion, L-DOPA produced diminishment in the percentage of the TH-ir neurons in comparison with controls, smaller still in comparison with the MPTP group. Despite its demonstrated potential to cause dopaminergic neurotoxicity when injected in the SNpc, L-DOPA produced a mild reduction of TH-ir neurons when compared with MPTP. The combined administration of MPTP and L-DOPA generated a similar dopaminergic

neurodegeneration inflicted by MPTP alone. In this context, we did not observe any immediate property of L-DOPA in the rescue of dopaminergic neurons from the MPTP insult.

Western blotting data showed that the striatal expression of TH protein was not altered in the groups tested. This response is consistent to Maeda and colleagues (1997), who reported no alteration in the relative density of striatal TH immunolabeling in the MPTP+L-DOPA group compared with the MPTP-saline group. In contrast, a significant reduction of TH expression in both L-DOPA and MPTP groups in the SN was verified when compared with controls. These data are suggestive of damage in the nigrostriatal pathway. Surprisingly, the MPTP+L-DOPA group did not exhibit a reduction of TH expression in the SN. That intriguing fact makes for compelling evidence of neuronal plasticity unchained by L-DOPA subsequent to the severe dopaminergic damage inflicted by MPTP. These data are in stark contrast to that encountered by Myers et al. (1999), which described that L-DOPA appears to enhance neuronal degeneration in animals the dopaminergic function of which has been compromised by neurotoxin assault.

The metabolism of L-DOPA and DA produces quinones, semiquinones, hydrogen peroxide, and other oxyradicals, which are believed to be toxic to the SN neurons (Fahn and Cohen 1992). Exogenous L-DOPA administration significantly decreased the DA levels within the striatum, corroborating previous data showing that high doses of L-DOPA are not accompanied by significant increases in extracellular DA; therefore, the observed effects would be due to the direct stimulation of DA receptors by the L-DOPA itself (Fisher et al. 2000). Such inference could be correct, because concentrations of the DA metabolites DOPAC and HVA were not found to be increased in the striatum.

The present results support earlier studies (Gross et al. 2003; Guigoni et al. 2005; Bendir et al. 2006; Konitsiotis and Tsironis 2006) indicating that L-DOPA alone is capable of inducing impairment in the dopaminergic system. In contrast, our results clearly demonstrated that the intranigral administration of L-DOPA, after previous MPTP infusion, presented a strong compensatory effect circumscribing motor behavior and TH expression parameters. Nevertheless, our neurochemical findings suggested that effect probably did not occur due to a consequence of L-DOPA, which provided additional supplies for DA synthesis in the neurons that survived from the MPTP lesion. Therefore, L-DOPA did not increase the striatal DA levels or DA metabolites, according to the adopted protocol. An alternative explanation could suggest a direct activation of dopaminergic receptors by L-DOPA, resulting in the compensatory effect. However, animals that received a single intranigral L-DOPA administration manifested a typical histological, behavioral, and neurochemical alteration of lesion of the nigrostriatal pathway.

It has been suggested that unlike the effects of L-DOPA in the normal SN, the drug may be toxic to the partially damaged SN neurons in PD patients (Rajput 2001). Nevertheless, we suggest that L-DOPA is more associated with a compensatory or even neuroprotective action when administered after a lesion. Thus, corroborating the work of Gross and colleagues (2003), we propose that L-DOPA may exert distinct effects on the dopaminergic system, mainly depending on the integrity of the nigrostriatal pathway. Future studies are warranted to complement the understanding of the interaction of L-DOPA therapy with the dopaminergic system in normal and in PD scenarios.

Conflicts of interest statement we declare that we have no conflict of interest.

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Chapter 22

Cannabinoid CB₁ Receptors are Early DownRegulated Followed by a Further UpRegulation in the Basal Ganglia of Mice with Deletion of Specific *Park* Genes

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Abstract This study was designed to examine the type of changes experienced by the CB₁ receptor, a key element of the cannabinoid signaling system, in the basal ganglia of different mouse mutants generated by deletion of specific genes associated with the development of Parkinson's disease in humans [*PARK1* (α -synuclein), *PARK2* (parkin) or *PARK6* (PINK1)]. We observed that CB₁ receptor-mRNA levels were significantly reduced in the caudate-putamen in the three models under examination when animals were analyzed at early phases (≤ 12 months of age). This decrease was, in general, associated with a reduction in CB₁ receptor binding in the substantia nigra and the globus pallidus, particularly in the case of α -synuclein-deficient mice. By contrast, both parameters, mRNA levels and binding for the CB₁ receptor, showed an elevation in the same areas when animals were analyzed at older ages, mainly in the case of the CB₁ receptor binding in the substantia nigra. In summary, our data revealed the existence of a biphasic response for CB₁ receptors, with losses at early phases, when dopaminergic dysfunction is possibly the major event that takes place, followed by upregulatory responses at advanced phases characterized by the occurrence of evident nigrostriatal pathology including neuronal death in some cases.

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Keywords Basal ganglia • Cannabinoid signaling system • CB₁ receptor • Parkin • Parkinsonism • PINK1 • α -synuclein

Introduction

Recent studies have demonstrated the existence of a close link between Parkinson's disease (PD) and the function of the cannabinoid signaling system in the basal ganglia (Sagredo et al. 2007, for review), sustaining the idea that cannabinoid-related compounds might serve as a novel therapy in this neurodegenerative disease (Gerdeman and Fernández-Ruiz 2008, for review). Thus, preclinical studies have described that selective antagonists of the cannabinoid type-1 (CB₁) receptor might be useful for the treatment of bradykinesia (Fernández-Espejo et al. 2005; González et al. 2006; García-Arencibia et al. 2008) and for delaying the development of levodopa-induced dyskinesia (Brotchie 2003, for review). By contrast, cannabinoid agonists have been proposed for the treatment of Parkinsonian tremor (Sañudo-Peña et al. 1999), given their well-demonstrated capability to reduce glutamate release and then to normalize glutamate overactivity in the subthalamic nucleus. In addition to these effects in alleviating specific Parkinsonian symptoms, cannabinoids, in particular those having antioxidant properties, have also demonstrated a notable capability to protect nigrostriatal neurons from death (Lastres-Becker et al. 2005; García-Arencibia et al. 2007).

The potential of the CB₁ receptor blockade for the treatment of bradykinesia finds a solid explanation in the overactivity experienced by the cannabinoid system in the basal ganglia in PD (Sagredo et al. 2007; Gerdeman and Fernández-Ruiz, 2008, for review). For example, studies of postmortem basal ganglia obtained from patients have demonstrated an increase in the number and function of CB₁ receptors in different basal ganglia structures (Lastres-Becker et al. 2001). Increases of endocannabinoid levels were measured in the cerebrospinal fluid of patients in another study (Pisani et al.

2005). Both observations were related to the development of Parkinsonism itself, since they tended to disappear with the treatment with levodopa (Lastres-Becker et al. 2001; Pisani et al. 2005). Overactivity of the cannabinoid system, reflected in an elevation of specific elements of this signaling system, has also been observed in different animal models of PD, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated marmosets (Lastres-Becker et al. 2001), 6-hydroxydopamine (6-OHDA)-lesioned rats (Romero et al. 2000; Gubellini et al. 2002), and reserpine-treated animals (Di Marzo et al. 2000). There is consensus that this overactivity of the cannabinoid system is directly associated with conditions of profound nigrostriatal pathology and existence of evident Parkinsonian symptoms, as occurred in the two studies conducted with samples from patients (Lastres-Becker et al. 2001; Pisani et al. 2005), or in the case of those experiments developed with animals lesioned with neurotoxins (Romero et al. 2000; Lastres-Becker et al. 2001; Gubellini et al. 2002). However, the type of changes experienced by the cannabinoid signaling might be different in early and presymptomatic stages of this disease, when nigral pathology is not evident or is still minimal. This has been observed in other neurodegenerative disorders, such as Huntington's disease (HD) (Sagredo et al. 2007; Pazos et al. 2008, for review) and amyotrophic lateral sclerosis (ALS) (Zhao et al. 2008), and sustains the idea that a malfunctioning of the cannabinoid signaling system, in particular at the level of CB₁ receptors, may be an early event in the pathogenesis of different chronic neurodegenerative disorders. This malfunctioning might be instrumental, since it might trigger excitotoxicity, inflammation, or other cytotoxic events that are normally under the control of CB₁ receptors (see Sagredo et al. 2007; Gerdeman and Fernández-Ruiz 2008, for recent reviews), indirectly supporting the idea that enhancing the cannabinoid signaling system might be neuroprotective in neurodegenerative disorders. This study was designed to provide support for this hypothesis by examining the pattern followed by CB₁ receptor binding and mRNA expression in the basal ganglia in the early-stage vs. late-stage pathology of various mouse mutants generated by deletion of specific genes associated with PD in humans: (1) *PARK1* (α -synuclein)-deficient mice developed by Cabin et al. (2002), (2) *PARK2* (parkin)-null mice developed by Itier et al. (2003), and (3) *PARK6* (PINK1)-deficient mice developed by Gispert et al. (unpublished data).

Materials and Methods

Animals and Sampling

Male *PARK1*^{-/-} (generated by Cabin et al. 2002), *PARK2*^{-/-} (generated by Itier et al. 2003), and *PARK6*^{-/-} (generated by

Gispert et al. unpublished data) mice, and their corresponding wild-type controls were bred in animal facilities of the University of Frankfurt (*PARK1*^{-/-} and *PARK6*^{-/-}) and in the "Hospital Ramón y Cajal" (*PARK2*^{-/-}) under controlled conditions of photoperiod, humidity and temperature, and with standard food and water available ad libitum (see details in Cabin et al. 2002; Rodríguez-Navarro et al. 2007; Gispert et al. unpublished data). All procedures using laboratory animals were in accordance with the European Union rules (directive 86/609/EEC). Animals were genotyped following standard PCR procedures (Cabin et al. 2002; Itier et al. 2003; Rodríguez-Navarro et al. 2007; Gispert et al. unpublished data). *PARK1*^{-/-} and their corresponding wild-type controls were killed at 12 months of age, whereas *PARK2*^{-/-} were killed at 4 or 18 months of age and *PARK6*^{-/-} at 9 and 24 months of age. After killing, brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice, and stored at -80°C for the evaluation of CB₁ receptor binding and mRNA levels.

Autoradiographic Analyses

Brain slicing. Coronal sections 20 μ m-thick were cut in a cryostat, according to Lehmann and Gautier (1974). The sections were thaw-mounted onto RNase-free gelatin/chrome alum-coated slides and dried briefly at 30°C and stored at -80°C until used. Adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet for the identification of the different brain nuclei.

Autoradiography of cannabinoid receptor binding. The protocol used was the method described by Herkenham et al. (1991) with slight modifications (González et al. 2005). Briefly, slide-mounted brain sections were incubated for 2.5 h, at 37°C, in a buffer containing 50 mM TRIS with 5% bovine serum albumin (fatty acid-free), at pH 7.4, and 10 nM [³H]-CP-55,940 (Du Pont NEN) prepared in the same buffer, in the absence or the presence of 10 μ M nonlabeled CP-55,940 (Sigma Chem., Madrid, Spain) to determine the total and the nonspecific binding respectively. Following this incubation, slides were washed in 50 mM TRIS buffer with 1% bovine serum albumin (fatty acid-free), at pH 7.4, for 4 h (2 $\times$ 2 h) at 0°C, dipped in ice-cold distilled water and then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labeled tissues, together with autoradiographic standards ([³H] microscases, Amersham), to tritium-sensitive film ([³H]-Hyperfilm, Amersham) for a period of 10 days. Autoradiograms were developed (D-19, Kodak) for 4 min at 20°C, and the films were analyzed in a computer-assisted videodensitometer using the curve generated from [³H]-standards.

Analysis of mRNA levels for CB₁ receptor by in situ hybridization.

The analysis of CB₁ receptor mRNA levels was carried out according to Rubino et al. (1994) with slight modifications (González et al. 2005). Briefly, sections were fixed in 4% paraformaldehyde for 5 min and, after rinsing twice in phosphate buffer saline, were acetylated by incubation in 0.25% acetic anhydride prepared in 0.1M triethanolamine/0.15M sodium chloride (pH 8.0), for 10 min. The sections were rinsed in 0.3M sodium chloride/0.03M sodium citrate, at pH 7.0, dehydrated and delipidated by ethanol/chloroform series. A mixture (1:1:1) of the three 48-mer oligonucleotide probes complementary to bases 4–51, 349–396, and 952–999 of the rat CB₁ receptor cDNA (Du Pont; the specificity of the probes used was assessed by Northern Blot analysis) was 3'-end labeled with [³⁵S]-dATP using terminal deoxynucleotidyl-transferase. Sections were then hybridized with [³⁵S]-labeled oligonucleotide probes (7.5×10^5 dpm per section), washed and exposed to X-ray film (Kodak) for 1 week, and developed (D-19, Kodak) for 6 min at 20°C. The intensity of the hybridization signal was assessed by measuring the grey levels in the autoradiographic films with a computer-assisted video-densitometer. Adjacent brain sections were cohybridized with a 100-fold excess of cold probe or with RNase to assert the specificity of the signal (data not shown).

Statistics

All numerical data were expressed as percentages over the corresponding values obtained in the wild-type controls and represent mean $\pm$ SEM. Differences between groups were evaluated using the Student's t-test.

Results

CB₁ Receptor Binding and mRNA Levels in α -Synuclein-Deficient Mice

Samples from α -synuclein-deficient mice were obtained at an age that, according to previous data (Cabin et al. 2002), represents major motor and neurochemical abnormalities such as reduced exploratory activity and low striatal dopamine contents, which are reminiscent of early Parkinsonian stages, but shows no evidence of neuronal degeneration. Unfortunately, these animals were not available at older ages. At this early age, α -synuclein-deficient mice showed a significant reduction in CB₁ receptor-mRNA levels in the

caudate-putamen (Fig. 1) that was accompanied by parallel, and more marked, reductions in CB₁ receptor binding in the substantia nigra and the globus pallidus (Fig. 1).

CB₁ Receptor Binding and mRNA Levels in Parkin-Deficient Mice

Samples from parkin-null mice were obtained at two ages that, according to previous data (Itier et al. 2003; Rodríguez-Navarro et al. 2007), represents two relevant stages in the progression of nigral pathology in these animals: (1) 4 month-old, an age at which the animals showed a series of motor and cognitive deficits and dopaminergic dysregulation (including reduced levels of dopamine transporter), but no evidence of neuronal death (see Itier et al. 2003); and (2) 18 month-old, an age characterized by a loss of dopaminergic neurons in the substantia nigra, increase in the number of proapoptotic proteins, reduced life span, and other signs of a profound nigral degeneration (Rodríguez-Navarro et al. 2007). Parkin-null mice, when analyzed at 4 months of age, showed again a significant reduction in CB₁ receptor-mRNA levels in the caudate-putamen (Fig. 1), although, in this case, this reduction was not accompanied by a parallel and statistically significant reduction in CB₁ receptor binding in the substantia nigra and the globus pallidus (Fig. 1). By contrast, when animals were analyzed at 18 months of age, they showed a significant increase in both CB₁ receptor-mRNA levels in the caudate-putamen (Fig. 2) and CB₁ receptor binding in the substantia nigra, with no significant effects in the globus pallidus (Fig. 2). Autoradiograms representative of the changes observed in CB₁ receptor-mRNA expression in the caudate-putamen of parkin-null mice in both ages can be seen in Fig. 3.

CB₁ Receptor Binding and mRNA Levels in PINK1-Deficient Mice

Samples from PINK1-deficient mice were obtained at two ages, 9 and 24 months of age, which represent the progression of dopaminergic dysregulation and mitochondrial pathology at two relevant stages. However, it should be noted that no neuronal death has previously been identified in these animals (Gispert et al. unpublished data). In this respect, we conducted some preliminary analysis of tyrosine hydroxylase (TH) immunoreactivity, with the purpose of identifying a possible loss of nigral neurons in 24 month-old animals, and found a subtle reduction in the number of TH-positive cells that did not exist in 9 month-old animals (unpublished data). This observation was concordant with the fact that PINK1-deficient mice, when analyzed at 24 months of age, showed significant increases in CB₁ receptor binding in the

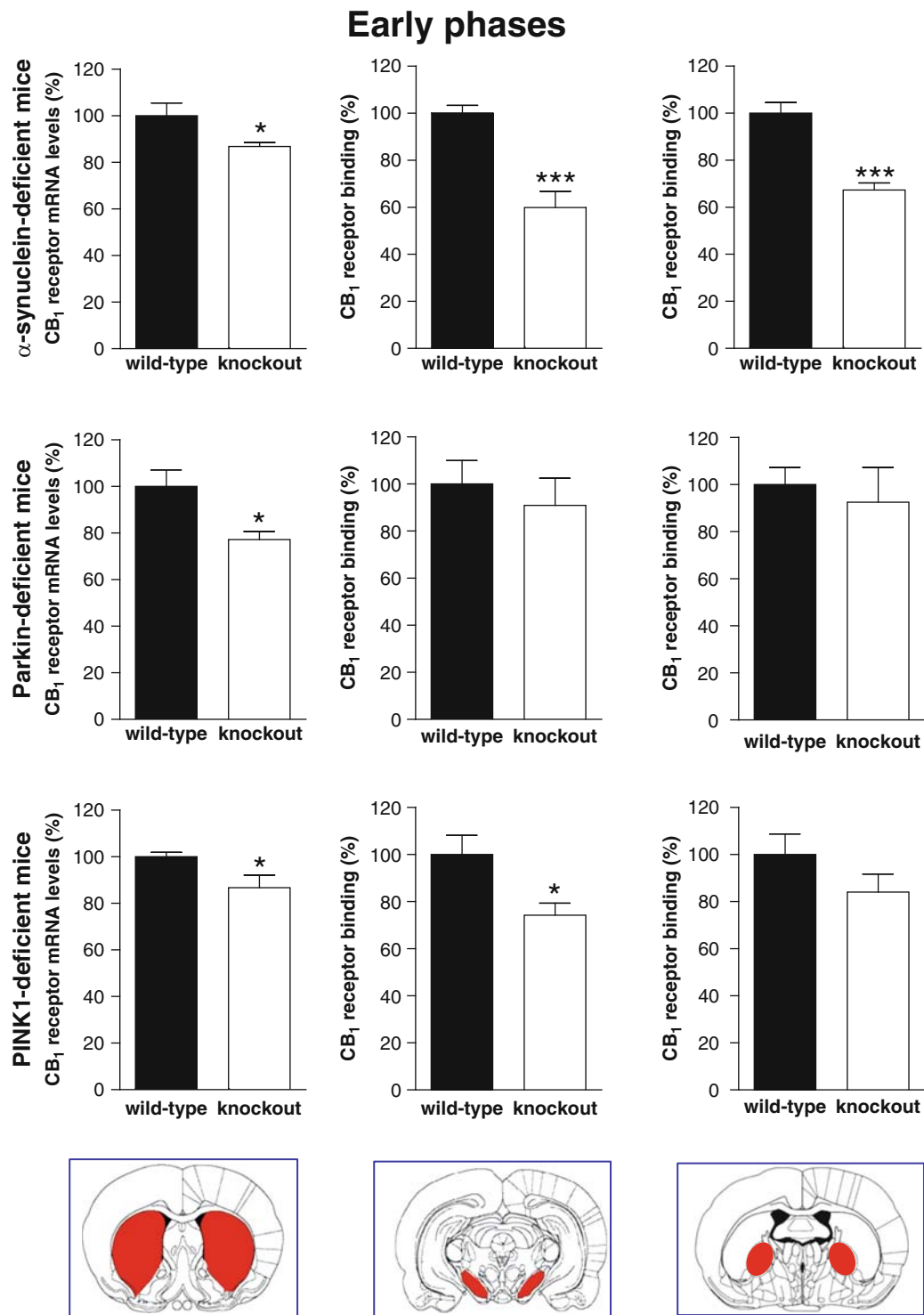


Fig. 1 CB₁ receptor binding and mRNA levels measured in the basal ganglia of mice with deletion of *PARK1* (α -synuclein), *PARK2* (parkin) or *PARK6* (PINK1) genes, and their corresponding wild-type controls, used at early ages (12 months for α -synuclein-deficient mice, 4 months for parkin-null mice and 9 months for PINK1-deficient animals). Data are percentages over the corresponding values obtained in the wild-type controls and represent mean $\pm$ SEM of 5–8 animals *per* group. Data were assessed by the Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

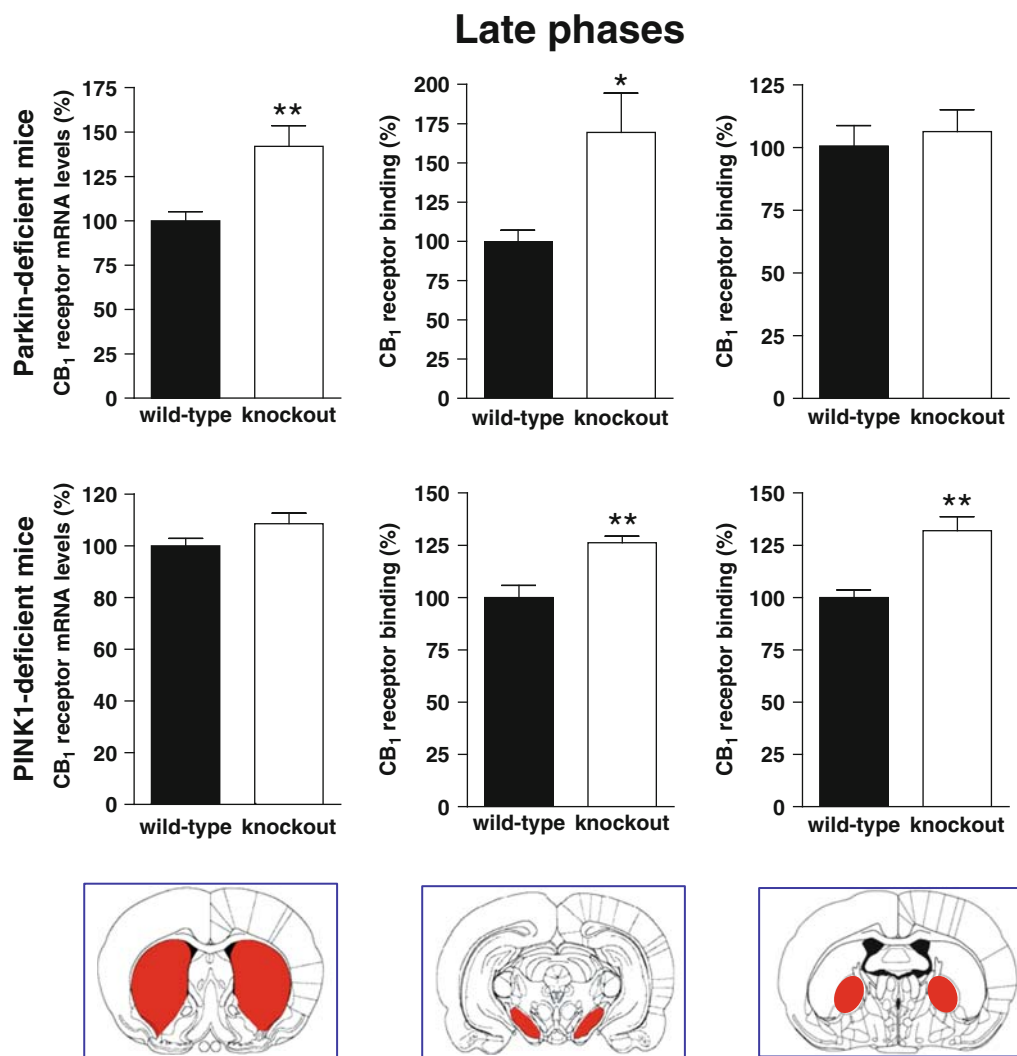
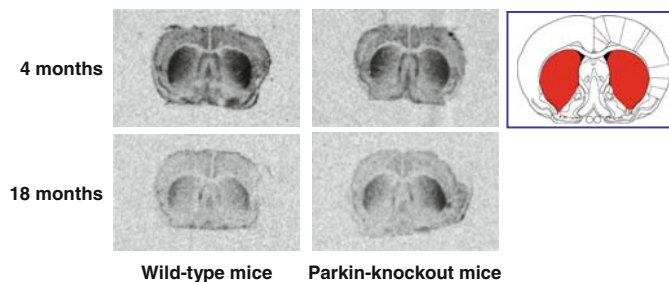


Fig. 2 CB₁ receptor binding and mRNA levels measured in the basal ganglia of mice with deletion of *PARK2* (parkin) or *PARK6* (PINK1) genes, and their corresponding wild-type controls, used at late ages (18 months for parkin-null mice and 24 months for PINK1-deficient animals). Data are percentages over the corresponding values obtained in the wild-type controls and represent mean $\pm$ SEM of 5–8 animals *per* group. Data were assessed by the Student's t-test (* p < 0.05, ** p < 0.01, *** p < 0.005)

In situ hybridization for CB₁receptor mRNA levels

Fig. 3 Representative autoradiograms used for the quantification by *in situ* hybridization of CB₁ receptor-mRNA levels in the caudate-putamen of mice with deletion of *PARK2* (parkin) gene and their corresponding wild-type controls, used at early (4 month-old) and late (18 month-old) ages



substantia nigra and the globus pallidus (Fig. 2) and a trend toward an increase in their mRNA levels in the caudate-putamen (Fig. 2), since these increases were representative of a situation of nigral pathology according to previous

(Romero et al. 2000; Lastres-Becker et al. 2001) and present data (e.g. parkin-null mice). By contrast, PINK1-deficient mice, when analyzed at 9 months of age, exhibited the typical downregulatory response found in the other models,

e.g., decreased CB₁ receptor-mRNA levels in the caudate-putamen (Fig. 1) and CB₁ receptor binding in the globus pallidus and, in particular, in the substantia nigra (Fig. 1).

Discussion

Most studies that have examined the changes produced in certain elements of the cannabinoid signaling system in the basal ganglia in PD are concordant with the idea that this signaling system becomes overactive as a consequence of the dopaminergic denervation of the striatum (Mailleux and Vanderhaeghen 1993; Romero et al. 2000; Di Marzo et al. 2000; Lastres-Becker et al. 2001; Gubellini et al. 2002; Pisani et al. 2005; González et al. 2006). This consensus has concentrated mainly on the CB₁ receptor, the upregulation/enhanced expression of which has been related to the typical hypokinetic profile of this disease, which also supported the proposed usefulness of CB₁ receptor antagonists for the treatment of Parkinsonian bradykinesia (Fernández-Espejo et al. 2005; González et al. 2006; García-Arencibia et al. 2008). This study has provided novel data to support this type of upregulatory responses, since they were evident in genetic mouse models of Parkinsonism when the animals were analyzed at ages at which incipient nigral pathology, including the degeneration of dopaminergic neurons, becomes evident, as in the case of *PARK2*^{-/-} (Rodríguez-Navarro et al. 2007) and to a lesser extent in *PARK6*^{-/-} (unpublished data) animals. However, it was hypothesized, and the present study has also provided experimental evidence to support this idea, that this change experienced by the CB₁ receptor and in general by the cannabinoid signaling system is not unique to the progression of PD. Data obtained in other neurodegenerative disorders, such as HD (Sagredo et al. 2007; Pazos et al. 2008, for review) and ALS (Zhao et al. 2008), suggested that the cannabinoid system might become hypofunctional in the first stages of these disorders, so that this downregulatory response might be instrumental in allowing the enhancement of a series of cytotoxic events that have been shown to be controlled, among other signals, by the cannabinoid activity (Gerdeman and Fernández-Ruiz 2008, for review). In this study, we have obtained strong evidence of these types of downregulatory responses consistently affecting the CB₁ receptors in the basal ganglia in the several mouse mutants of *PARK* genes at early stages when the only abnormalities detected were certain motor deficits and dopaminergic dysregulation, but no neuronal death. This is an important point, since it indicates: (1) that the decrease in CB₁ receptor might be an early event presumably involved in the initiation and/or progression of PD, (2) that it might be considered as a biomarker for studies in humans, and

(3) that the activation of this receptor might have neuroprotective properties. A loss/malfunctioning of CB₁ receptors might be involved in the development of excitotoxicity, since this receptor type has been involved in the inhibition of glutamate release (Gerdeman and Fernández-Ruiz 2008), but it might also elicit other cytotoxic responses like oxidative stress and inflammation, although these responses have been related more to other type of cannabinoid signals (Sagredo et al. 2007; Gerdeman and Fernández-Ruiz 2008).

There is a last aspect of this study that deserves some additional comments. In Fig. 3, one may appreciate the existence of a significant loss in the levels of CB₁ receptor transcripts in young wild-type mice when compared with old wild-type animals. This reduction, however, was significantly lower in the case of their corresponding mutant animals, potentially explaining the biphasic response found for this parameter in *PARK2*^{-/-} mice, and in a similar way in the other models and for the other parameters. Although this interpretation is tempting and in fact is supported by previous studies of the effects of aging on CB₁ receptors in healthy rats (Romero et al. 1998; Berrendero et al. 1998), we cannot confirm that this may be a convincing explanation for the results found here. In our study, the comparison between data in young and aged animals was not possible, because, given the extreme differences in age, the data in young animals were obtained in assays and with analytical conditions (different specific activity for labeled oligonucleotide probes, times of exposure, etc.) different from in the case of old animals, so that a multiple comparison assessed by the two-way analysis of variance was not possible.

In summary, our data revealed the existence of a biphasic response for CB₁ receptors, with losses at early ages in these mice, when dopaminergic dysfunction is possibly the major

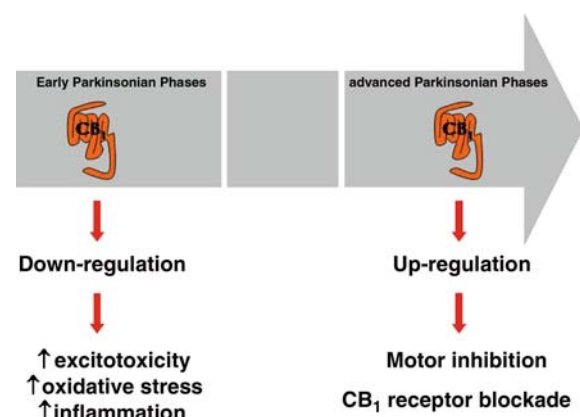


Fig. 4 Model proposed for the physiopathological consequences of the biphasic responses of CB₁ receptors observed during the progression of pathology in mouse mutants generated by deletion of *PARK1* (α -synuclein), *PARK2* (parkin) or *PARK6* (PINK1) genes

event that takes place, followed by upregulatory responses at the late ages characterized by the occurrence of evident nigrostriatal pathology including neuronal death in some cases (see Fig. 4 for a diagram summarizing the biphasic response found and their proposed physiopathological consequences).

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 23

Neurogenesis in Substantia Nigra of Parkinsonian Brains?

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Abstract The clinical motor dysfunction in Parkinson's disease is primarily the consequence of a progressive degeneration of dopaminergic neurons in the substantia nigra of the nigrostriatal pathway. The degeneration of this tract provokes a depletion of dopamine in the striatum, where it is required as a permissive factor for normal motor function. Despite intense investigations, no effective therapy is available to prevent the onset or to halt the progression of the neuronal cell loss. Therefore, recent years have seen research into the mechanisms of endogenous repair processes occurring in the adult brain, particularly in the substantia nigra. Neurogenesis occurs in the adult brain in a constitutive manner under physiological circumstances within two regions: the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles. In contrast to these two so-called neurogenic areas, the remainder of the brain is considered to be primarily nonneurogenic in nature, implying that no new neurons are produced there under normal conditions. The occurrence of adult neurogenesis in the substantia nigra under the pathological conditions of Parkinson's disease, however, remains controversial. Here, we review the published evidence of whether adult neurogenesis exists or not within the substantia nigra, where dopaminergic neurons are lost in Parkinson's disease.

Keywords Dopamine • Neurogenesis • Parkinson's disease • Regenerative medicine • Stem cells • Substantia nigra

Abbreviations

6-OHDA	6-hydroxydopamine
BDNF	Brain-derived neurotrophic factor
BrdU	5-bromo-2'deoxyuridine
GDNF	Glia-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
LGF	Liver growth factor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NeuN	Neuronal nuclei antigen
NSCs	Neural stem cells
OB	Olfactory bulb
PCNA	Proliferating cells nuclear antigen
PD	Parkinson's disease
PDGF-BB	Platelet-derived growth factor-BB
PSA-NCAM	Polysialylated neural cell adhesion molecule
RMS	Rostral migratory stream
SGZ	Subgranular zone
SNC	Substantia Nigra pars compacta
SNr	Substantia Nigra pars reticulata
SVZ	Subventricular zone
TGF α	Transforming growth factor-alpha

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Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder rising in incidence after the age of 60. Clinically, PD is characterized by progressive motor symptoms (bradykinesia, resting tremor, rigidity, and postural instability), mainly linked to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc), which leads to the depletion of dopamine in the

striatum, the main target of the axonal projections arising from the SNc. Despite the availability of effective symptomatic drugs, there is presently no cure for PD, and all attempts to slow the neuronal cell loss in the disease have failed (Rascol et al. 2000).

Novel cell-based therapies aiming at a stimulation of endogenous dopamine production at a constant rate within the brain might provide a more physiological and elegant way to overcome the dopaminergic deficiency in Parkinsonian brains.

Initial enthusiasm for an experimental cell replacement therapy by grafting fetal neuronal precursor cells into the striatum has vanished after two double-blind placebo-controlled clinical trials showing only moderate symptomatic improvement and the occurrence of severe disabling dyskinesia (Freed et al. 2001).

Neural stem cells (NSCs) derived from adult tissues are an attractive alternative source for cell therapy for PD. They overcome the ethical issues inherent to the use of human fetal tissue or embryonic stem cells. NSCs derived from adult tissue also open the possibility for autologous transplantations without immunological complications, where NSCs are taken out from the patient, expanded, differentiated, and reimplanted back as dopaminergic precursor cells (Arias-Carrión and Yuan 2009).

The discovery of constitutively ongoing neurogenesis in the adult human brain has challenged the traditional view of a fixed circuitry in functionally normal brains and has raised high hopes that the adult brain may have the capacity for self-renewal after injury, thereby avoiding the need for transplantation (Borta and Höglinger 2007). Primary neural precursor cells reside in specialized zones called “neurogenic niches” (Alvarez-Buylla and Lim 2004). A population of NSCs preserves enough germinal character to maintain neurogenesis throughout life, and once differentiated, their daughter cells integrate into already existing neuronal networks (Alvarez-Buylla and Lim 2004; Zhao et al. 2008). Whether adult neurogenesis can be induced under certain circumstances in regions that lack constitutive adult neurogenesis remains controversial, but several studies have reported the isolation of NSCs from different regions of the adult brain, including the SNc (Arias-Carrión et al. 2007).

Interestingly, many studies using animal models of brain diseases have convincingly shown that in the adult brain, the “neurogenic niches” respond to insults by producing new progenitor cells, which migrate to sites that have been affected by neurodegenerative pathology or brain injury (Curtis et al. 2007a). However, it remains controversial whether adult NSCs do possess the capacity to produce functional dopaminergic neurons in PD or in corresponding animal models (Arias-Carrión et al. 2007). As this controversy is still unsettled, we review the published evidence in favor or against the existence of adult neurogenesis in the adult SNc.

Methodological Considerations for Studying Neural Stem Cells in vivo

There are many methodological issues to consider while assessing adult neurogenesis in vivo.

One issue is the method of identifying proliferating NSCs and their daughter cells. The earliest studies used [³H]-thymidine, which gets incorporated into the DNA during the S-phase of the cell cycle, labels dividing cells and their progeny in vivo and can be detected by autoradiography (Altman and Das 1965; Cameron et al. 1993).

The introduction of the synthetic thymidine analog 5-bromo-2'deoxyuridine (BrdU) substitutes for thymidine in newly synthesized DNA of proliferating cells (Kuhn et al. 1996; Eisch and Mandyam 2007). BrdU incorporated into DNA can then be easily visualized by immunohistochemistry using specific anti-BrdU antibodies. With this method, it is possible to perform a quantitative analysis of proliferation, phenotypic differentiation, and survival of newborn cells by varying the time interval between the pulsed administration of BrdU and the perfusion of animals (Eisch and Mandyam 2007). Using this approach, one can estimate the number of cells in S phase at a particular time if brains are examined at a relatively short time (e.g. 1 h) after a BrdU pulse; proliferating cells can be tracked through several anatomical divisions, when examined several days or weeks after a BrdU pulse. Even though BrdU labeling is considered to be the gold standard for the detection of adult neurogenesis, it has several pitfalls. To provide unambiguous data for neurogenesis, one must demonstrate BrdU-co-localization with phenotypic markers in three orthogonal planes using confocal microscopy. Additionally, sources of false positive phenomena need to be excluded (mitosis-like apoptosis, DNA repair, fusion of neurons with proliferating glia, DNA endo-replication) (Arias-Carrión et al. 2007). Although DNA labeling by BrdU is currently the most commonly used method for studying adult neurogenesis, the potential toxic effect of this thymidine analog should not be ignored, as it might be a confounding factor in some experiments.

This has led to the use of other markers of the cell cycle to analyze cell proliferation in situ, such as proliferating nuclear antigen (PCNA) and Ki-67 (Eisch and Mandyam 2007; Arias-Carrión et al. 2007). However, since the expression of these markers is turned off after the termination of the mitotic cell cycle, the phenotypic differentiation of the newborn daughter cells can not be tracked.

To overcome this drawback, retroviral transfection may be used for labeling mitotic cells by forced expression of green fluorescent protein (GFP) or other reporter genes, thus allowing a complete morphological or electrophysiological analysis of the daughter cells (Eisch and Mandyam 2007).

A second methodological issue concerns the criteria for unambiguous identification of a cellular phenotype. Currently, the neuronal nuclei antigen (NeuN) is the standard immunocytochemical marker to identify mature neurons. Glial fibrillary acidic protein (GFAP) and S100 β are used as markers for mature astrocytes (Eisch and Mandyam 2007). However, at present there are no undisputed marker proteins that can provide unambiguous prospective identification of intermediate precursor cells in the adult brain (Lledo et al. 2006).

Neurogenesis in the so-called Neurogenic Areas

The dogma that the adult mammalian brain is incapable of generating new neurons has been finally overcome, since neurogenesis in the adult brain was described in the subventricular zone (SVZ) adjacent to the lateral ventricles and in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Alvarez-Buylla and Lim 2004).

The SVZ contains the largest pool of NSCs in the adult mammalian brain, including the human brain (Curtis et al. 2007a, b; Zhao et al. 2008). In rodents, the adult SVZ contains four distinct cell types defined by morphological, ultrastructural, and molecular markers and electrophysiological properties: (1) neural progenitor (type A) cells express PSA-NCAM, Tuj1, and Hu; (2) NSCs in the SVZ are protoplasmic astrocytes (type B cells) and express nestin, vimentin, and GFAP; (3) transit amplifying (type C) cells are nestin positive and form clusters adjacent to the chains of migrating neuroblasts throughout the SVZ; and 4) ependymal ciliated (type E) cells separate the SVZ from the ventricular cavity. The organization of the adult human SVZ is significantly different from that of rodents. In adult rodents, SVZ astrocytes (Type B cells) are located next to the ependymal layer (Alvarez-Buylla and Lim 2004). In contrast, in the adult human brain SVZ astrocytes are not found adjacent to the ependymal cells and no chains of migrating neuroblasts are found in this region (Sanai et al. 2004). Instead, the cell bodies of human SVZ astrocytes accumulate in a band or ribbon separated from the ependymal layer by a gap that is largely devoid of cells (Sanai et al. 2004). Neural progenitors migrate from the SVZ through the rostral migratory stream (RMS), and after reaching the olfactory bulb (OB), they originate olfactory granule and periglomerular interneurons (Alvarez-Buylla and Lim 2004; Zhao et al. 2008). Granule neurons are all GABAergic, whereas only 40% of the periglomerular neurons are GABAergic, and of those, 65% are also dopaminergic (Zhao et al. 2008). Under normal conditions, neural progenitors in the SVZ remain as nondifferentiated nonexcitable cells. They begin to display properties of mature interneurons only on their arrival to the

olfactory bulb (Alvarez-Buylla and Lim 2004; Zhao et al. 2008). However, in humans, the extent of SVZ neurogenesis and the existence of the RMS are controversial (Sanai et al. 2004; Curtis et al. 2007a, b).

In the hippocampus, NSCs are localized in the SGZ, situated between the *hilus* and the granular layer of the dentate gyrus (Alvarez-Buylla and Lim 2004; Zhao et al. 2008). Astrocytes (type B cells) give rise to progenitor cells (type D cells; neuroblasts), which differentiate into new granule cells (type G cells). During maturation and differentiation, newly generated cells migrate through the granule-cell layer and get integrated into the basic circuitry of the hippocampus through synaptic contacts with pyramidal neurons in the CA3 field (Alvarez-Buylla and Lim 2004; Zhao et al. 2008).

Neurogenesis in the so-called Nonneurogenic Areas

By definition, neurogenic areas require the presence of both NSCs and a neurogenesis-permissive microenvironment. Environmental signals in the neurogenic niches appear to depend on both histological peculiarities and specific local soluble signals to control the neurogenic behavior of the NSCs throughout adult life (Arias-Carrión et al. 2007; Alvarez-Buylla and Lim 2004). Populations of NSCs, which unfold their neurogenic potential once explanted and stimulated with appropriate cues *in vitro*, have been reported to exist in normal adult cortex, amygdala, dorsal vagal complex of the adult brainstem, area CA1 of the hippocampus, spinal cord, striatum, white matter tracts, and SNc (Arias-Carrión et al. 2007). Even though the generation of neurons from NSCs *in vivo* in these regions has been alleged (Arias-Carrión et al. 2007), the majority of the available data support the view that the potential of the NSCs is restricted by inhibitory cues in these brain areas, thus defining them as being primarily nonneurogenic in nature. Therefore, despite the presence of cells with neurogenic potential, primarily nonneurogenic areas of the adult mammalian brain appear to exhibit strong inhibitory cues to prevent neurogenesis from occurring under physiological conditions *in vivo* (e.g. Kornack and Rakic 2001; Lie et al. 2002; Bhardwaj et al. 2006; Höglinger et al. 2007).

Importantly, however, it has been reported that specific damage to primarily nonneurogenic regions results in the proliferation of local NSCs and the neuronal differentiation of their progeny. For example, NSC proliferation in the neocortex was observed in a rodent model of selective pyramidal cell apoptosis, leading to differentiation into mature neurons with establishment of axonal connections to the

appropriate target sites in remote brain areas (Magavi et al. 2000). Similarly, targeted apoptosis of corticospinal motor neurons has been reported to induce neurogenesis with an extension of axons into the spinal cord over 3–4 months (Chen et al. 2004). Lesion experiments in the hippocampus (Nakatomi et al. 2002) and the striatum (Arvidsson et al. 2002) also provided support to the concept of lesion-induced neurogenesis in normally nonneurogenic regions. These data inspired optimism for the reparative potential of endogenous NSCs in the context of a diseased adult brain.

Controversial Evidence for Neurogenesis in Substantia Nigra of Parkinsonian Brain

The substantia nigra (a midbrain structure) is considered part of the basal ganglia complex due to its close ties with the striatum (Arias-Carrión and Pöppel 2007). Classically, it has been divided into two components: the pars compacta (SNc) and the pars reticulata (SNr). The SNc is a cell-rich region that in humans is composed of large pigmented dopaminergic neurons. Cells in the SNc exhibit a triangular/fusiform shape and have traditionally been divided into two types (Björklund and Dunnett 2007). First, small neurons (10–12 μ m soma diameter) with short axons are considered to be the major nigral interneurons. In rodents, they represent about 10% of all neurons in the SNc. The second type of neuron is larger (15–20 μ m soma diameter) and constitutes the nigral output neurons.

The clinical motor dysfunction in PD is primarily linked to the depletion of dopamine in the striatum consecutive to the loss of the large dopaminergic neurons in the SNc. The classical view is that this is the result of a progressive degeneration process triggered by unidentified pathogenic factors. This hypothesis was challenged in 2001 by Armstrong and Barker (2001), who proposed the concept that the dopaminergic population in the SNc undergoes a continuous turnover with a low rate of spontaneous neuronal cell loss and an equilibrated rate of constitutive neurogenesis to replace the lost neurons. According to this concept, the reduced number of dopaminergic neurons in the SNc in PD would result from a reduced rate of adult neurogenesis rather than by an increased rate of neuronal cell loss (Armstrong and Barker 2001). In 2003, Zhao and coworkers presented experimental findings that suggested that new dopaminergic neurons would indeed be continuously born in the SNc of adult mice to replace spontaneously degenerating neurons. According to this hypothesis, the dopaminergic neuronal population in the SNc would undergo a complete turnover during the lifespan of a mouse (Zhao et al. 2003). Since then, the hypothesis that the generation of new dopaminergic neurons in the adult SNc could occur spontaneously or as

a consequence of lesion-induced repair-mechanisms has received much attention.

Cells expressing polysialylated neural cell adhesion molecule (PSA-NCAM), a sensitive marker of immature neuroblasts, have been detected in the postmortem substantia nigra of human PD patients, (Yoshimi et al. 2005). This has been put forward as an argument for ongoing neurogenesis in the adult human SNc. However, PSA-NCAM is also expressed in reactive astrocytes (Oumesmar et al. 1995; Nomura et al. 2000) and in neurites of mature neurons undergoing plastic changes (Charles et al. 2002). Thus, these findings do show that some degree of cellular plasticity is maintained in the SNc, even in aged human PD patients, but they are no strong evidence in favor of neurogenesis in the adult human substantia nigra in PD.

In the adult rodent SNc, the existence of cells expressing the EGF-receptor has been demonstrated (Seroogy et al. 1994), which characterizes rapidly proliferating neural precursor cells (C-Cells) in the SVZ (Höglinger et al. 2004). Indeed, the adult rodent midbrain appears to contain progenitor cells with the potential to differentiate into astrocytes, oligodendrocytes, and neurons, particularly dopaminergic neurons, once these precursor cells are explanted and grown in vitro (Reynolds and Weiss 1992; Lie et al. 2002; Hermann et al. 2006).

However, it remains controversial whether these precursor cells can unfold their neurogenic potential within the parenchyma of the adult brain. There are some reports describing BrdU⁺ neurons with dopaminergic cytoplasm in the substantia nigra of rodents, which occurred either spontaneously or even more pronouncedly after an experimentally induced degeneration of the nigrostriatal dopaminergic projection. So far, four articles have reported the existence of in vivo neurogenesis in the adult SNc of experimental animals (Zhao et al. 2003; Van Kampen and Robertson 2005, 2006; Shan et al. 2006).

Zhao and coworkers used several (Fig. 1) methods to provide evidence in favor of an ongoing turnover of the dopaminergic neuronal cell population in the rodent SNc (Zhao et al. 2003). These authors described a constitutive nigral neurogenesis, and in addition, an increased neurogenesis in response to an MPTP-induced lesion of the nigrostriatal neurons. The authors suggested that the newly generated cells are derived from stem cells lining the cerebroventricular system. Unfortunately, the illustrations presented leave a degree of uncertainty about the newborn nature of the dopaminergic neurons, since the colocalization of a tyrosine hydroxylase (TH)⁺ cytoplasm around the BrdU⁺ nucleus is not unequivocally demonstrated in all three dimensions.

Van Kampen and Robertson (2005) reported that on average, 10 BrdU⁺ cells can be found in the substantia nigra of healthy adult female rats, of which 15 % coexpressed the neuronal marker NeuN and 8% the catecholaminergic

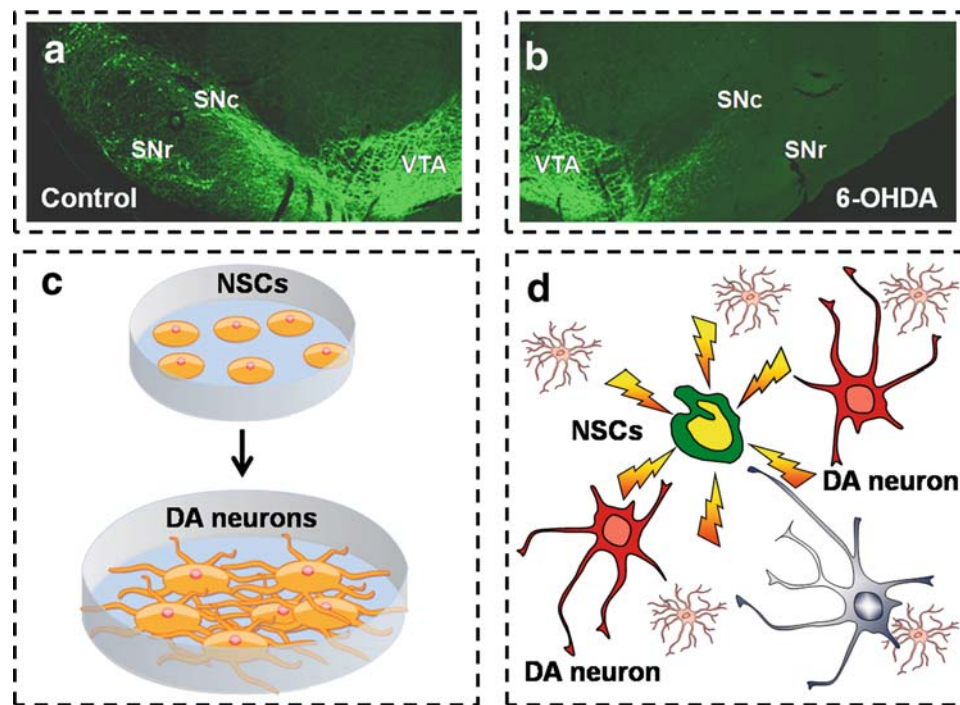


Fig. 1 (a) The ventral midbrain in a rat, stained immunohistochemically for tyrosine hydroxylase (green), comprises the substantia nigra pars compacta (SNc) containing predominantly dopaminergic neurons giving rise to nigrostriatal axons, the substantia nigra pars reticulata (SNr) containing mainly GABAergic neurons and some dopaminergic neurons, and the ventral tegmental area (VTA) containing mainly dopaminergic neurons giving rise to mesolimbic axons. (b) In Parkinson's disease and corresponding animal models (here exemplified by a 6-OHDA-lesioned rat), a preferential degeneration of the dopaminergic neurons in the substantia nigra with relative sparing of the VTA is observed. (c) In the parenchyma of the normal adult ventral midbrain, presence of neural stem cells (NSCs) has been reported, which unfold their neurogenic potential once explanted and stimulated with appropriate cues in vitro. (d) In vivo, however, the neurogenic potential of the adult parenchymal NSCs in the ventral midbrain appears to be restricted by presently unidentified inhibiting cues (flashes), which might be emitted for example by local glial cells or mature neurons, rendering the substantia nigra in the adult brain a primarily non-neurogenic area

marker TH. These findings imply that a low rate of physiological neurogenesis seems to occur in the rodent SNc. By a chronic infusion of 7-OH-DPAT, a D3 receptor agonist, the total number of BrdU⁺ cells was reported to increase to approximately 70 cells on average, of which 30% coexpressed NeuN and 20% TH. The authors concluded that D3 receptor stimulation would induce a strong precursor cell proliferation and would promote the expression of a dopaminergic phenotype in the newborn neurons.

One year later, Van Kampen and Eckman (2006) published an increase of BrdU/NeuN/TH triple labeled cells also in a 6-OHDA rat model of PD, when animals were treated with 7-OH-DPAT. In contrast to Zhao et al. (2003), Van Kampen and Eckman (2006) proposed a local parenchymal origin of the newborn cells based on the absence of colabeling with doublecortin, a marker for migrating neuroblasts. In the confocal pictures provided in this publication, the nuclear marker BrdU and the cytoplasmic marker TH are stained in the same cellular compartment, suggesting the rupture of the nuclear membrane, which is a typical feature of apoptotic cell death (Vila and Przedborski 2003) or antibody 'bleeding' (Kornack and Rakic 2001). Thus, it is uncertain whether

the authors have indeed depicted genuinely newborn neurons or perhaps rather preexisting neurons undergoing a form of mitosis-like apoptosis, which has been demonstrated to occur in the 6-OHDA and MPTP models of PD and in human PD patients (El-Khodori et al. 2003; Höglinger et al. 2007). It might therefore be that the increase in the number of TH⁺ nigral neurones in 6-OHDA lesioned rats after 7-OH-DPAT treatment, reported by Van Kampen and Eckman, does not represent neurogenesis, but rather other mechanisms of cellular plasticity such as phenotypic recovery or phenotype shifting (Tandé et al. 2006).

Shan and coworkers reported findings suggestive of constitutive levels of neurogenesis, in particular dopaminergic neurogenesis, in mice (Shan et al. 2006). This phenomenon was reported to increase after MPTP-induced dopaminergic depletion (Shan et al. 2006). In their experiments, the authors used brain slices with 12µm diameter, which makes a correct interpretation of their findings impossible, since the nigral cell diameter can be up to 15µm (Van Kampen and Robertson 2005). Thus, according to the rules of stereology, a newborn dopaminergic neuron with a BrdU⁺ nucleus can by no means be distinguished from a BrdU⁺ nucleus of an

astrocytic satellite cell invaginated into a preexisting dopaminergic neuron (Borta and Höglinger 2007). Besides, in the pictures shown, the BrdU staining is stronger in the cytoplasm than in the nucleus, suggesting that the BrdU incorporated into the nuclear DNA reflects an incorporation of a small number of BrdU molecules into the DNA strand as occurs during DNA repair rather than a comprehensive DNA duplication during mitotic cell division.

Furthermore, several independent groups have tried unsuccessfully to obtain evidence of generation of BrdU⁺ dopaminergic neurons in the adult rodents' substantia nigra in an unlesioned condition (Kay and Blum 2000; Mao et al. 2001; Lie et al. 2002; Frielingsdorf et al. 2004; Chen et al. 2005; Mohapel et al. 2005; Reimers et al. 2006; Aponso et al. 2008) or after experimental degeneration induced by 6-OHDA (Lie et al. 2002; Frielingsdorf et al. 2003; Cooper and Isacson 2004; Mohapel et al. 2005; Reimers et al. 2006; Steiner et al. 2006; Aponso et al. 2008) or by MPTP (Kay and Blum 1994; Mao et al. 2001; Chen et al. 2004; Höglinger et al. 2007).

Even the additional exposure to factors known to increase neurogenesis in the SVZ or the SGZ, such as transforming growth factor- α (TGF α), glia-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor-BB (PDGF-BB), liver growth factor (LGF), or housing in an enriched environment and physical activity, did not lead to a detectable rate of neurogenesis in the SNc in an in vivo condition (Arias-Carrión et al. 2007). It has been reported that an increase in the number of dopaminergic nigral neurones can be induced by the administration of GDNF (Chen et al. 2005). This effect, however, resulted from an upregulation of dopaminergic neuronal markers in already existing neurons rather than from the generation of new neurones (Chen et al. 2005).

Conclusions

In recent years many studies have provided evidence for the presence of NSCs in the neurogenic areas of the adult brain. The NSCs have the potential to generate new neurons, including dopaminergic neurons. Intriguingly, such NSCs are present not only in brain areas known to be permissive for adult neurogenesis in a constitutive way, but also in more widespread areas of the brain, such as the SNc, that are thought to be primarily nonneurogenic in nature. Despite the presence of precursor cells that show neurogenic potential when explanted in a cell culture setting, adult nigral neurogenesis in vivo seems rather unlikely to occur under physiological and pathological conditions. The presently available data do not demonstrate unequivocally that an injury of the nigrostriatal dopaminergic projection is per se

sufficient to trigger adult dopaminergic neurogenesis in the SNc. Future work will have to address the important question of whether manipulations of the local cues in the micro-environment of the adult SNc will release the potential of endogenous stem or precursor cells to replace the lost neurons in an anatomically and functionally appropriate way.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 24

Stem Cells and Cell Replacement Therapy for Parkinson's Disease

K.-C. Sonntag, F. Simunovic, and R. Sanchez-Pernaute

Abstract Parkinson's disease (PD) is a neurodegenerative disorder caused by a progressive degeneration of the midbrain dopamine (DA) neurons in the substantia nigra pars compacta (SNc) that predominantly affects the ventral population projecting to the dorsal striatum and leads to a gradual dysfunction of the motor system. There is currently no cure for PD. Pharmacological and surgical (e.g. deep brain stimulation) interventions can alleviate some of the symptoms, but lose their efficacy over time. The distinct loss of DA neurons in the SN offers the opportunity to assay neuronal cell replacement, and the clinical transplantation of fetal midbrain neuroblasts in PD patients has shown that this approach is feasible. However, there are multiple problems associated with the use of fetus-derived material, including limited availability. DA neurons derived from stem cells (SC) represent an alternative and unlimited cell source for cell replacement therapies. Currently, human pluripotent SC, such as embryonic (ES), and most recently, induced pluripotent stem cells (iPS), and multipotent (tissue-specific) adult SC are available, although the methodology for a reliable and efficient production of DA neurons necessary for biomedical applications is still underdeveloped. Here, we discuss some essentials for SC and SC-derived DA neurons to become therapeutic agents.

Keywords Adult Neurogenesis • Cell Replacement Therapy • Dopamine • Fetal Midbrain • Parkinson's Disease • Stem Cells

Abbreviations

6-OHDA 6-hydroxydopamine
AADC Aromatic L-amino acid decarboxylase

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BMP	Bone morphogenic protein
DA	Dopamine
DAT	Dopamine transporter
ESC	Embryonic stem cells
FACS	Fluorescent activated cell sorting
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
iPS	Induced pluripotent stem cells
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
SC	Stem cells
SHH	Sonic hedgehog
SNc	Substantia nigra, pars compacta
SNCT	Somatic nuclear cell transfer
SVZ	Subventricular zone
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
VMAT-2	Vesicular monoamine transporter-2

Introduction

Parkinson's disease (PD) is a severe neurodegenerative disorder caused by the loss of the dopamine (DA) neurons in the substantia nigra (SN). In the US, there are currently 1.5 million people with PD, and every year about 60,000 new patients are diagnosed (The Michael J. Fox Foundation for Parkinson's disease Research, <http://www.michaeljfox.org/>). Sporadic PD develops after age 65 (15% of PD patients develop symptoms before the age of 50) and affects both men and women – there are sparse data on gender differences but the incidence is greater in male than female (Baldereschi et al. 2000; Cantuti-Castelvetri et al. 2007). The cause of sporadic PD is unknown, but a combination of genetic and environmental factors appears likely. In the past decade, genetic linkage and association analyses have identified several loci associated with familial forms of the disease (PARK 1–13) and mutations have been found in

familial monogenic forms of the disease in seven genes so far. Although these inherited forms account for less than 10% of the cases (reviewed in Belin and Westerlund 2008), mutations in some of the PARK genes appear to increase the risk of developing sporadic PD, pointing to shared disease mechanisms and pathways (Moran et al. 2007; Thomas and Beal 2007; Burke 2008). However, it is not known how genetic alterations lead to the loss in the SN of DA neurons that cause the typical clinical manifestations. In addition, symptoms appear late in the course of the disease process, i.e., after death or damage of 60–80% of the approximately 1 million nigral DA neurons. Therefore, protective or preventive therapies may not be sufficient. To date, available treatments include chemical replacement therapy (L-DOPA and direct and indirect DA agonists), antioxidant agents, growth factors, deep brain stimulation, and others (Sonntag et al. 2005; Benabid 2007; Melamed et al. 2007; Schapira 2007). These therapies can improve some of the symptoms, but do not stop PD from progressing and some of these treatments are associated with side effects such as involuntary movements, tics, hallucinations, confusion, and dramatic swings in mobility and mood (Koller and Tse 2004). A different approach aims to restore cell function by replacing the lost DA neurons and the feasibility of this new therapeutic intervention has been demonstrated with fetal midbrain neuroblasts that can integrate in patient's brains after transplantation and alleviate the symptoms of PD (Lindvall and Bjorklund 2004; Hall et al. 2007). However, there are several practical and ethical issues associated with this approach including low efficiency, variability in the clinical outcome, and ethical controversy regarding the use of fetal cells (Lindvall 2000; Bjorklund et al. 2003; Lazic and Barker 2003; Drucker-Colin and Verdugo-Diaz 2004; Lindvall and Bjorklund 2004; Hall et al. 2007). Pluripotent stem cells (SC) appear to be a better cell source for biomedical applications because of their unlimited availability and amenability to in vitro manipulation and standardization (Isacson 2003; Lindvall 2003; Sonntag et al. 2005; Sonntag and Sanchez-Pernaute 2006; Yu and Thomson 2008). The best-characterized SC population so far are embryonic stem cells (ESC) including those from the human species, which can be differentiated into DA neurons that function in animal models of PD (Ben-Hur et al. 2004; Buytaert-Hoefen et al. 2004; Park et al. 2004; Perrier et al. 2004; Schulz et al. 2004; Zeng et al. 2004; Yan et al. 2005; Brederlau et al. 2006; Roy et al. 2006; Iacovitti et al. 2007; Ko et al. 2007; Sonntag et al. 2007; Cho et al. 2008; Hong et al. 2008). However, there are several problems associated with this approach, such as ethical issues, difficulties in controlling cell differentiation, instability of the generated DA phenotype, teratoma formation, and allogeneity, which can lead to graft rejection in clinical

settings (Li et al. 2008a). Other cell sources comprise multipotent (tissue-specific) adult SC and induced pluripotent SC (iPS). Although these cells have been less characterized than ESC, they could offer the advantage of serving as a patient-specific cell source. Altogether, there are several possibilities to generate DA neurons from SC, each with advantages and limitations (Fig. 1a), and it is currently unclear which one might enter the clinical arena. Here, we discuss the different SC sources in light of their potential to fulfill the requirements for producing DA neurons as therapeutic agents for PD.

Fetal Midbrain-Derived Cells

Fetal midbrain-derived cells are obtained from aborted embryos at the time of DA neurogenesis between 6 and 9 weeks of gestational age. Based on the experimental evidence of functional recovery of motor deficits induced by fetal midbrain transplants in Parkinsonian rodents, clinical studies were initiated in 1987. Several clinical open trials followed with variable outcome. Due to prominent methodological differences, a meta-analysis of those studies is difficult to conduct, but a moderate clinical improvement (about 30% in the motor subscale of the UPDRS), significant enhancement of F-DOPA uptake in the putamen and postmortem evidence of graft survival and synaptic connections with host neurons have been reported (Freed et al. 1992; Lindvall 1994; Freeman et al. 1995; Kordower et al. 1996; Hagell et al. 1999; Piccini et al. 1999, 2000). Some factors accounting for the clinical variability include patient's age and the number of fetal donors, donor tissue preparation (i.e. minced solid pieces, dissociated cell suspensions, or mesencephalic tissue strands (Clarkson et al. 1998)), immunosuppression, and placement (putamen, caudate, nigra, or multiple sites (Mendez et al. 2002)). Despite some improvements in earlier studies, the results of two double blind trials conducted in the US (Freed et al. 2001; Olanow et al. 2003) showed limited, age-dependant benefit, as well as unexpected side effects, and thus, brought attention to additional factors that complicate graft outcome, in particular from the host, such as age, L-DOPA responsiveness and long-term L-DOPA therapy. Subsequent studies have revealed further aspects, such as the activation of local inflammatory reaction (Mendez et al. 2005) and the heterogeneity of graft cell composition, including a high proportion of serotonin neurons (Mendez et al. 2008). Experimental data suggest that the presence of serotonin neurons in the grafts can contribute to the development or worsening of L-DOPA-induced dyskinesias (Carlsson et al. 2007; Carta et al. 2007). Recently, the description of Lewy bodies (i.e. proteinaceous aggregates, which have

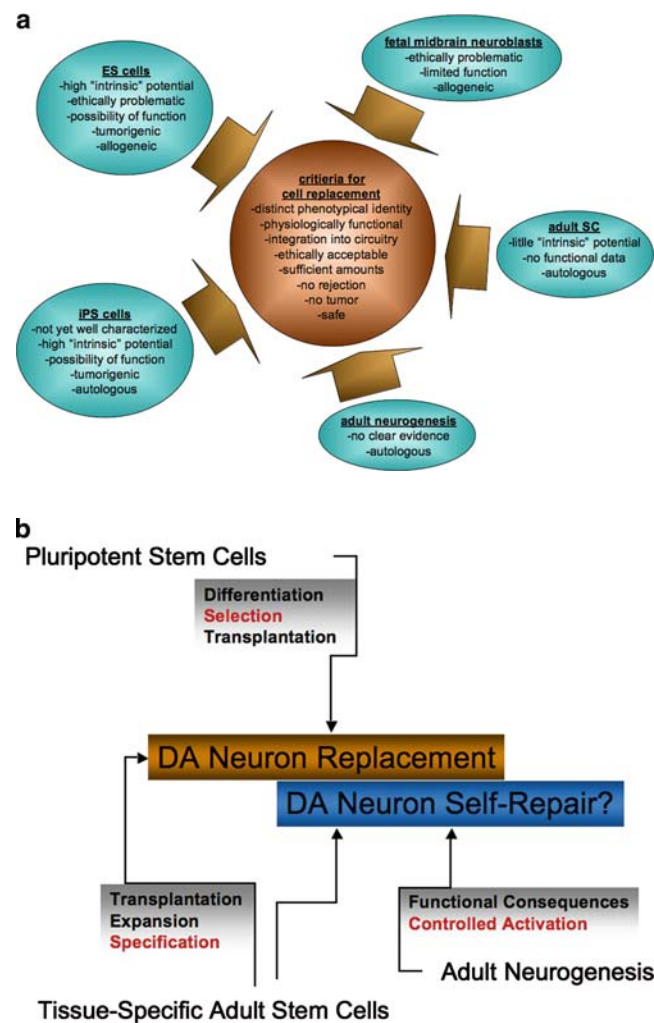


Fig. 1 Criteria and currently investigated SC sources and approaches for potential cell replacement therapy in PD. **(a)** Several criteria need to be fulfilled to render SC a safe and efficient source for restoring midbrain DA neuronal function. Clinical transplantation of fetal midbrain neuroblasts “paved the way” for this approach and patient surveillance demonstrated its feasibility and potential. But this approach has ethical and technical problems including immune responses to the graft. Alternative autologous restoration of cell function in the SN could be achieved by endogenous adult neurogenesis, or through implantation of differentiated adult SC. However, the therapeutic potential of these cell sources has yet to be demonstrated. In contrast, pluripotent SC seem to be a potent source for production of DA neurons, which could be derived from ESC and more recently also from iPS. Although some function in animal models was reported, these experiments also pointed to many obstacles regarding their survival, integration into the host circuitry, and remaining potential of tumor formation. In addition, there is currently very little information on how this approach will be translated to potential application in humans taken in account the more advanced brain complexity and the higher potential of immune responses – although the latter could be circumvented by “patient-customized” iPS. **(b)** Schematic depiction of stem cell-based approaches to functional restoration in PD described in this review. Highlighted in red in the text boxes are the most pressing challenges for each strategy: selection of the desired population from pluripotent SC, correct specification for adult SC and controlled activation of proliferation for the endogenous neural precursor

a high content of alpha-synuclein and are regarded as the pathological hallmark of PD) in grafted neurons (Kordower et al. 2008; Li et al. 2008b) has led to a reconsideration of the feasibility of functional neuronal cell replacement for PD. So far, it is unclear why young DA neurons from different genetic backgrounds would develop alpha-synuclein aggregates after 10 years. A number of studies suggest that Lewy bodies in PD may represent a defensive response of the cell (Chen and Feany 2005) such as in other degenerative

diseases in which misfolded or misassembled proteins are sequestered in aggregates. There is convincing evidence from a wealth of recent studies that the soluble oligomers or fibrils are cytotoxic, while the development of inclusions appears to be consistently protective in different cellular models. A reasonable approach is to consider that replacing the lost neurons in PD is only part of the solution, which will necessarily have to integrate other measures directed to prevent the supracellular pathological process from affecting the new neurons.

Another important issue refers to the cell type that is needed for functional restoration. Fetal midbrain grafted cells are most likely to be acting in the host brain through several mechanisms, whereas only a small proportion of the DA neurons present in the grafts are able to reestablish synaptic connections and regulate axonal release of DA (Lindvall and Bjorklund 2004). Synapses are specialized contacts between two specific partners and require bidirectional recognition and communication (Cohen-Cory and Lom 2004). Thus, only cells that display a specific midbrain DA phenotype will be able to make the necessary connections with the medium-size striatal spiny neurons. Synaptic reconnection should prevent the occurrence of side effects, such as dyskinesias, which is a typical manifestation of deregulated DA transmission. In fetal midbrain grafts very few cells (probably less than 5%) are DA neurons. Moreover, in the midbrain there are several DA subpopulations (mesoprefrontal, mesocorticolimbic, and mesostriatal) that display different anatomical, electrophysiological, and molecular properties (Lammel et al. 2008). The exact contribution to the beneficial and adverse clinical effects of these and other non-DA midbrain cell populations that form the graft is at present unclear. The possibility of deriving homogeneous neuronal and other neural cell populations from stem cells should help to clarify individual contributions and define optimal cell compositions to maximize functional restoration (Thompson et al. 2008).

Stem Cells for Neurogenesis and DA Specification

An alternative cell source to fetal cells are SC, which have the advantage of being available in unlimited amounts and – depending on their source and derivation – are not necessarily associated with ethical limitations. In addition, the generation of cell populations from SC could not only offer a cell source for replacement and treatment, but could also be a tool for studying cell function and the effects of factors involved in dysfunction and degeneration. The SC sources currently under investigation are pluripotent (Yu and Thomson 2008), i.e., fertilized and parthenogenic embryonic (ESC), induced pluripotent SC (iPS), and multipotent (tissue-specific) adult SC.

SC development depends on a strictly organized network orchestrating the chronological sequence of processes that integrate intracellular and environmental factors, which provide a temporal, positional, and molecular framework to achieve normal cell development. For a specific cellular phenotype to be generated *in vitro*, this environment needs to be recreated (Sonntag and Sanchez-Pernaute 2006). A key

aspect in deriving neural cell populations from pluripotent SC refers to their differentiation according to major steps in normal neurogenesis. This includes the generation of neural precursor populations, followed by differentiation along the neural lineages (Sonntag et al. 2005; Dang and Tropepe 2006; Sonntag and Sanchez-Pernaute 2006; Wilson and Stice 2006; Zhang 2006). Neurulation, i.e., the development of the neural tube, occurs in late gastrulation after the formation of the three germ layers, endo-, meso-, and ectoderm and the mesodermal notochord. The neural tube forms from the neuroectoderm and gives rise to the central and peripheral nervous systems. It organizes itself into a long-stretched structure with an anterior–posterior (forebrain-, midbrain-, and hindbrain) and ventral–dorsal axial orientation, which directs organogenesis of the embryo. The acquisition of cell fates in (vertebrate) neurogenesis involves many factors and mechanisms that interact in a complex spatiotemporal manner and can be summarized in four sections: neural induction and specification, anteroposterior regional character, neurogenesis, and neuronal identity (Diez del Corral and Storey 2001). In each of these steps, several key signaling pathways play a major role in cell fate determination (for summary see, e.g. Wilson and Edlund 2001; Lazzari et al. 2006; Lupo et al. 2006; Wilson and Stice 2006; Zhang 2006).

A critical aspect in neurogenesis refers to the function of members of the transforming growth factor- β (TGF- β) superfamily, which play an important role in cell morphogenesis and lineage specification in the developing brain (Schier and Shen 2000; Munoz-Sanjuan and Brivanlou 2001, 2002). The regulation of neuronal growth, differentiation, and specification depends on the activity of these growth and morphogenetic factors, their antagonists, and target membrane-bound receptors and intracellular signaling proteins (Tiedemann et al. 1998; Munoz-Sanjuan and Brivanlou 2002). In several animal models, such as frog (*Xenopus*), zebrafish, chick, and more recently in mouse, a default mechanism of neural induction has been postulated (Harland 2000; Wilson and Edlund 2001; Munoz-Sanjuan and Brivanlou 2002). This model proposes that neural induction is initiated by the inhibition of BMP signaling in the embryonic ectoderm through the normal activity of BMP inhibitors.

The development of midbrain DA neurons also depends on a complex interplay of multiple factors and some of them have been identified as essential for their generation, survival, and maintenance (Alavian et al. 2008). Key molecules in the early steps of ventral midbrain development as well as the induction of DA precursors are the signaling and growth factors sonic hedgehog (SHH), Wnt1, and fibroblast growth factor 8 (FGF8). These work together with a set of transcriptional activators, such as *Msx1*, *Lmx1/2*, *Pitx3*, *Engrailed-1/2*, *FoxA2*, and *Nurr1*, which – in concert with other molecules – specify the mesencephalic DA phenotype (Alavian et al.

2008). These factors are not only functional in a spatiotemporal manner in normal embryogenesis, but can also be used in *in vitro* and *in vivo* model systems to produce midbrain-like DA neurons (Chung et al. 2002; Kim et al. 2002; Andersson et al. 2006; Martinat et al. 2006). Thus, the knowledge about normal neurogenesis and DA cell specification has been instrumental in developing *in vitro* protocols for SC differentiation toward functional DA neurons.

Embryonic Stem Cells (ESC)

Pluripotent ESC are derived from the blastocyst and have the potential to develop into any cell type *in vitro* and *in vivo*. In the past decade, extensive knowledge about cell biology, genetic manipulation, and *in vitro* culture methods has made ESC a powerful tool to generate multiple cell populations that could function in model systems of disease (Murry and Keller 2008). In addition, ESC differentiation paradigms could be used to study normal (and abnormal) cell development and to delineate intrinsic and extrinsic pathways as well as the influence of the environment on cell differentiation. The potential of differentiated DA neurons as therapeutic agent in PD has originally been pioneered in mouse and primate ESC, demonstrating their survival, differentiation, and morphological properties, as well as function, as demonstrated in rat and primate animal models of PD (Deacon et al. 1998; Bjorklund et al. 2002; Kawasaki et al. 2002; Kim et al. 2002; Sanchez-Pernaute et al. 2005; Sonntag et al. 2005; Takagi et al. 2005; Sanchez-Pernaute et al. 2008). In recent years, this approach has also been translated to human ESC, demonstrating the derivation of DA neurons that show many features of the DA phenotype (Ben-Hur et al. 2004; Buytaert-Hoefen et al. 2004; Park et al. 2004; Perrier et al. 2004; Schulz et al. 2004; Zeng et al. 2004; Yan et al. 2005; Brederlau et al. 2006; Roy et al. 2006; Iacovitti et al. 2007; Ko et al. 2007; Sonntag et al. 2007; Cho et al. 2008; Hong et al. 2008) and some functional survival in a rat model of PD (Ben-Hur et al. 2004; Roy et al. 2006; Sonntag et al. 2007; Cho et al. 2008; Li et al. 2008a). Although these studies demonstrated the feasibility of the ESC paradigm, there are also major problems attached, which diminished the initial enthusiasm for this approach. These mainly refer to the difficulty of controlling cell differentiation toward a homogeneous DA cell population based on their intrinsic pluripotent potential and of achieving cultures devoid of contaminating immature cells that can give rise to teratoma formation after transplantation into Parkinsonian animals (e.g. Schulz et al. 2004; Brederlau et al. 2006; Roy et al. 2006; Sonntag et al. 2007). In addition, some cultures generated incorrect or instable DA phenotypes, which demonstrated poor survival and suboptimal function *in vivo*

(Sonntag and Sanchez-Pernaute 2006). This could be attributed to their incomplete characterization, a transient expression of the tyrosine hydroxylase (TH) gene, and/or a “DA-like” phenotype that was derived from neural crest or other neural precursors. Altogether, these data indicated that more research is necessary to improve the quality and quantity of transplantable cells in the ESC paradigms. Several protocol improvements and additional approaches have been published (summarized in Li et al. 2008a), which include a recently reported increase of the DA neuronal cell populations up to ~80% (Cho et al. 2008). In addition, it appears that longer *in vitro* differentiation cultures could diminish the fraction of immature precursors and abrogate teratoma formation (Ben-Hur et al. 2004; Cho et al. 2008). Other approaches comprise cell-sorting technologies to enrich for specific or eliminate unwanted cell populations, such as fluorescent activated cell sorting (FACS) (Chung et al. 2006; Hedlund et al. 2007, 2008; Pruszek et al. 2007; Li et al. 2008a).

ESC represent a powerful tool for understanding *in vitro* neurogenesis and the production of functional DA neurons. However, it is currently unclear whether this cell source will enter the clinical arena, mainly because of the ethical and technical limitations of their generation and the difficulties in producing a homogeneous population of phenotypically correct midbrain-like DA neurons.

Induced Pluripotent SC (iPS)

Other than ethical issues, a major concern using ESC and their derivatives is their allogeneic nature, which renders them potential targets for rejection when implanted into the CNS and might compromise their survival in PD patients (Li et al. 2008a). Possible solutions to overcome rejection processes were implicated by “customized” SC through therapeutic cloning using somatic nuclear cell transfer (SNCT) or cell fusion, which could result in fewer immunogenic transplants (Lanza et al. 2002; Hwang et al. 2005). However, these approaches were burdened with a number of obstacles, most notably high technological requirements, complex procedures, inadequate efficiency of stem cell generation and their developmental potential and ethical concerns (Jaenisch and Young 2008). A new method for not only customizing but also creating SC is based on “nuclear reprogramming” to induce pluripotent SC (iPS or iPSC) and there is great hope that this technology will provide researchers and clinicians with patient-specific SC lines (Park et al. 2008) that can be used for scientific, diagnostic, or therapeutic purposes, including the production of DA neurons for PD. The development of iPS was pioneered by Takahashi and Yamanaka in 2006, who expressed four SC-specific transcription factors

(Oct3/4, Sox2, c-Myc and Klf4) in somatic mouse cells, rendering them ESC-like and pluripotent (Takahashi and Yamanaka 2006). Since this revolutionary discovery of “turning back” mature cells into an immature state, this field of research has been expanding at a great pace. So far, many improvements have been made to make this process more efficient and safer, such as eliminating the oncogene Myc (Nakagawa et al. 2008) or even reducing the number of factors to two (Oct4 plus Klf4 or c-Myc) (Kim et al. 2008). Furthermore, the improvements in the selection mechanism to avoid drug selection led to greater efficacy of iPS generation, facilitating the work with various cell lines (Blelloch et al. 2007; Meissner et al. 2007). Most importantly, the differentiation of mouse iPS using standard ESC differentiation protocols demonstrated their ability to differentiate into neural cells including multipotent neural precursors giving rise to glia and specified neurons, such as DA neurons (Takahashi and Yamanaka 2006; Wernig et al. 2008). The therapeutic potential of iPS was also demonstrated in a mouse model of sickle cell anemia (Hanna et al. 2007), and recently, in the rodent 6-OHDA model of PD (Wernig et al. 2008) attesting to their potential as therapeutic agent. Also patient-specific pluripotent SC lines could already be generated from human fibroblasts (Takahashi and Yamanaka 2006; Yu et al. 2007; Dimos et al. 2008). However, this technology has to undergo extensive testing before its potential introduction to clinical application. A gene or protein delivery system alternative to the commonly used retroviral vector system will need to be developed, because viral delivery involves an unacceptably high risk of mutation and carcinogenesis. Finally, iPS seem to behave like ESC and their differentiation is associated with similar problems, such as the presence of lingering immature cells that can give rise to teratoma formation. Nevertheless, iPS cells remain one of the most exciting developments in modern biomedicine already showing great promise in bringing patient-specific cell treatment closer to reality.

Adult SC

In contrast to pluripotent ESC and iPS, adult – or somatic – SC are multipotent and are obtained from the adult organism. They are found in many organ systems (including but not limited to, bone marrow, fat tissue, testes, muscle, gut, skin or brain) where they maintain tissue homeostasis and contribute to a certain extent to the replenishment of lost or damaged cells (Rando 2006). Adult neural SC show great promise for restorative approaches (see below, under Endogenous adult neurogenesis), but they are not easily accessible and therefore efforts have been directed to derive neurons from other, more readily available, adult SC sources.

Mesoderm-derived cells are the most abundant and well-characterized source of adult SC and despite their incompatible lineage determination with ectoderm-derived neural cells, in vitro differentiated mesenchymal SC demonstrated some plasticity, and could be redirected to form neuronal cell populations (Kondo et al. 2005; Wislet-Gendebien et al. 2005) including DA neurons (Jiang et al. 2003; Guo et al. 2005; Fu et al. 2006; Tatard et al. 2007). Adult SC are defined by two properties: self-renewal in the undifferentiated state and a lineage-limited differentiation potential that gives rise to cell types of the tissue or organ from which they originate. Thus, the differentiation potential of adult SC does not follow the early steps of embryogenesis, which include the formation of cells from the three germ layers. However, studies of adult SC suggest the existence of a substantial degree of plasticity that allows for differentiation into phenotypes across germ-layers known as “transdifferentiation” (Jackson et al. 2007; Phinney and Prockop 2007; Eberhard and Tosh 2008). Conceptually, transdifferentiation is based on the idea that lineage-determined stem or progenitor cells retain their ability to differentiate into multiple cellular phenotypes within and across their embryonic germ layer origin. This concept is still controversial in the field of SC research, mainly because of the increasing use of technologically advanced differentiation systems that complicate the distinction between “natural” and “artificial” cell development. For example, under defined in vitro differentiation conditions, using gene-engineering technologies, SC populations derived from the adult system can be forced to differentiate into mature cells that they normally would not form in their respective in vivo environment (Krabbe et al. 2005; Vieyra et al. 2005). It is important to note that, in contrast to iPS, which are generated by similar paradigms, adult SC are not “reprogrammed” first into a more immature cell. In general, adult stem cells follow developmental rules, and accordingly, can be instructed to generate those lineages that arise later but not earlier during embryogenesis. There is currently little evidence that transdifferentiated mesenchymal SC can develop into a full and normal mature DA phenotype that can function in model systems of disease (Dezawa et al. 2004; Suon et al. 2006; Ye et al. 2007). Despite this rather disappointing outcome, adult SC offer major advantages over other SC sources for cell therapy. If used as an autologous cell population, the problem of immunogenicity could be avoided. Moreover, the ethical and legal issues are not nearly as controversial as those that burden the embryonic and fetal-derived SC sources. However, so far no efficient and methodologically feasible differentiation protocol has been developed to produce functional DA neurons from adult SC. This will be a crucial step before testing these cells in animal models of disease, hence moving the adult stem paradigm one step closer to clinical application.

Finally, it appears that adult stem cells may be easier to reprogram into iPS than terminally differentiated adult cells and may therefore provide an interesting autologous cell source in the near future. In particular, adult neural stem cells have recently been shown to require just two factors (Kim et al. 2008) to be reprogrammed, probably because of their high endogenous expression of Sox2, one of the key factors involved in the reprogramming process.

Endogenous Adult Neurogenesis

An alternative strategy with potential to replace midbrain DA neurons other than the implantation of cells could be the activation of the brain's own capacity to regenerate (Fig. 1b). Until recently, it was generally accepted that in the mammalian brain neurogenesis (i.e. the production of new neurons) ceases perinatally and that the adult brain possesses no intrinsic regenerative capacity. However, research over the last decade revealed that the human brain has a persisting reservoir of precursor cells and that these cells have the capacity to divide and give rise to differentiated cells including neurons (Gage 2000; Colucci-D'Amato and di Porzio 2008). In mammals, it is now well established that neurogenesis persists at least at two sites in the adult brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus. These regions maintain a distinct niche that promotes the survival and renewal of precursor cells. Neuroblasts born in these regions appear to have defined destinies (Alvarez-Buylla and Garcia-Verdugo 2002): SVZ neural precursors travel along the rostral migratory stream and finally reach the olfactory bulb, where they become granule and periglomerular neurons and these precursors demonstrate a remarkable fate restriction (Merkle et al. 2007). Subgranular neurons remain in the hippocampus, migrate in the dentate gyrus and become granule neurons (Zhao et al. 2008). Neural stem cells have also been isolated from other brain regions, but it is unclear whether they originated at neurogenic sites and migrated to target regions, were present as senescent precursors, or were even generated in other parts of the brain. In any case, adult neurogenesis appears to be implicated in diverse neurological processes, such as olfaction, mood regulation, cognition, memory and learning, and in the pharmacological effect of antidepressants, as well as in various neurological disorders (Zhao et al. 2008). Therefore, the concept of controlled stimulation of intrinsic neurogenesis is clinically appealing and has also been considered for potentially replacing DA neuron loss in PD. However, whether neurogenesis in the SN and striatum persists in the adult organism is a controversial issue. In rodents, the presence of neural precursors was demonstrated

in the SN (Lie et al. 2002) and these cells, when isolated and cultured *in vitro*, could be propagated and matured into DA neurons (Hermann et al. 2006). In addition, the stimulation of D3 receptors with a D3 DA agonist could recover DA neurons and induce neurogenesis in the adult SN (Van Kampen and Robertson 2005, 2006). Some other investigators have postulated a persistent neurogenesis in the adult SN (Zhao et al. 2003; Yoshimi et al. 2005; Shan et al. 2006) suggesting that, under a slow but constant neuronal turnover rate, the entire DA population in the SN would be replaced during the lifespan of a mouse (Zhao et al. 2003). However, these findings have been extensively challenged, as many investigators failed to find DA neurogenesis in the nigrostriatal system (Kay and Blum 2000; Frielingsdorf et al. 2004; Chen et al. 2005; Mohapel et al. 2005). Altogether, there is no clear evidence for endogenous adult neurogenesis other than in the SVZ and dentate gyrus (and maybe cerebellum), and intrinsic neurogenesis is unlikely to play a major role in PD pathogenesis.

Another related aspect that has received considerable attention is the DA modulation of adult neurogenesis (Borta and Hoglinger 2007; Geraerts et al. 2007). During embryogenesis, DA regulates proliferation and differentiation of striatal precursor cells with opposing effects exerted through D1 (reduce) and D2 (induce) DA receptor activation (Popolo et al. 2004). In the adult SVZ, afferent DA projection fibers have been identified and the origin of these projections was traced back to SNc. Consistently, several types of SVZ neural precursors were shown to express DA receptors and experimental ablation of the DA system in the MPTP and 6-OHDA rat models caused a marked reduction of cell proliferation in the SVZ (Hoglinger et al. 2004), while treatment with D2-like agonists stimulated their proliferation (Van Kampen et al. 2004). These findings are in line with neuropathological postmortem examinations, which described a reduction in the number of proliferating precursors in the SVZ of PD patients (Hoglinger et al. 2004). If there is, in addition to the degenerative loss of SN DA neurons, a certain reduction in precursor proliferation in PD, this could influence potential therapeutic strategies. A small boost in intrinsic neurogenesis by pharmacological agents, like D3 agonists, might be clinically relevant, because only 2–4% of the DA innervation to the striatum needs to be restored to achieve a significant improvement of PD motor symptoms (Brundin et al. 1994; Mohapel et al. 2005). However, such effects are difficult to define, because D3 agonists also cause a symptomatic benefit. In addition, a caveat is warranted. The understanding of why neurogenesis persists in adulthood is still limited. The artificial stimulation of neurogenesis could have adverse effects in that overstimulation might grow large numbers of precursors, which migrate, may or may not differentiate, and insufficiently, or even aberrantly, integrate in the local circuits, and disturb

residual function. This has been exemplified by recent experimental evidence showing that the stimulation of adult neurogenesis leads to prolonged seizures in animal models of temporal lobe epilepsy (Scharfman and Hen 2007). Furthermore, there is increasing experimental evidence that high-grade astrocytomas and gliomas may arise from precursors or stem cells in the SVZ (Galli et al. 2004; Kwon et al. 2008), and therefore, these approaches should be regarded with caution.

Requirement on SC and SC-Derived DA Neurons as Therapeutic Agents for PD

Independent of the therapeutic strategy that might enter future clinical application in the treatment of PD, intrinsic or extrinsic DA neuronal cell replacement has to fulfill strict conditions on both cell production and on their function in patients. Regarding the *in vitro* differentiation of SC, one needs to keep in mind that any differentiation protocol is artificial and can only incompletely recapitulate normal cell development. This is especially true if one considers that the majority of the *in vitro* systems are two-dimensional, unlike the three-dimensional structure of the developing embryo. Also, nonphysiological concentrations of factors are applied to the cultures. This also includes gene-engineering approaches, which are used to exogenously overexpress or inhibit factors and can compromise the genomic integrity and/or force the developing cells into “unnatural” physiological states (Sonntag et al. 2004). Thus, under nonphysiological and highly artificial conditions, normal cell development is not necessarily warranted and can lead to abnormal cell types that are suboptimal as a source for experimental and therapeutic paradigms. To render SC and SC-derived DA neurons therapeutic reagents for the treatment of PD, several requirements need to be met.

Requirements on the SC Source

Besides specific needs on the differentiation of each individual SC source, there are also common requirements (Li et al. 2008a). A paramount necessity is their inability to form tumors, such as teratoma or excessive outgrowth of distinct cell populations, which has mainly been observed with the use of pluripotent SC (Brederlau et al. 2006; Roy et al. 2006; Sonntag et al. 2007; Li et al. 2008a). The causes of tumor formation are multifactorial. The most common is the intrinsic ability of SC to self-renew indefinitely and produce large amounts of precursor cells that differentiate along multiple

cell lineages. Other reasons refer to the transformation of SC and their derivatives causing their development into tumor-like cells. This can be spontaneous or triggered by, e.g., gene-engineering (Haviernik and Bunting 2004; Li et al. 2008a). SC should be easily accessible, available in a sufficient amount and, if possible, patient-matched. For all SC sources currently under investigation, these requests are only partially met. For example, although sufficient amounts of pluripotent ESC are available, there are many ethical and technical problems involved in producing patient-customized cell lines. iPS cell technology addresses some of these issues, but this field is still in its infancy and it is too early to predict the therapeutic potential of this cell source. Patient-derived adult SC could have some advantages over ESC or iPS, such as reduced immunogenicity and potentially less need for manipulation. However, many hurdles need to be overcome to make these cells easily accessible and produce sufficient amounts of therapeutically functional cell populations. Finally, the differentiation of SC should not include the use of animal-derived components. This especially refers to coculture with cells from other species, e.g., mouse fibroblast or stromal cells, which bear the potential of contaminating the SC with unwanted reagents, such as endogenous retroviruses (Weiss 2006; Denner 2008).

Requirements on the DA Neuron

The optimal scenario for reconstructive cell replacement in PD would be the implantation of a cell population that fully restores the damage caused by a loss of the SN DA neurons. As already discussed, the feasibility of this approach has been demonstrated in cell transplantation paradigms using fetal midbrain neurons in clinical PD, and to some extent, SC-derived DA neurons in animal models of PD. However, for the latter, many obstacles toward a safe and functional therapy still remain. A key aspect is the production of a (homogeneous) DA cell population that has a stable and phenotypically distinct identity, that is available in sufficient amounts, does not form tumors, will not be rejected in the host brain, is physiologically functional, and integrates into the nigrostriatal circuitry. To restore synaptic connections and a regulated DA neurotransmission, donor cells need to have the molecular machinery to produce, store, and release DA (i.e. TH, AADC, GTP-cyclohydrolase, VMAT-2, etc.). Importantly, they need to express D2 autoreceptors and the DA transporter (DAT) to tightly control the extracellular levels of DA, cellular excitability, and basal levels of intracellular calcium. In the past few years, it has become evident that the expression of regional and cell-specific transcription factors such as Nurr1 (Saucedo-Cardenas et al. 1998), Pitx3 (Smidt et al. 1997), engrailed (EN-1 and -2) (Alberi et al.

2004; Sgado et al. 2006; Sonnier et al. 2007), and Foxa2 (Kittappa et al. 2007) is required for the survival of DA neurons in the adult brain. Additionally, trophic factors such as GDNF (Pascual et al. 2008) are also critical to the survival of DA neurons in the adult brain and may need to be provided concomitantly.

Conclusions

Given the complexities of disease progression and systemic involvement observed in PD patients, DA neuronal replacement alone may not be sufficient. Better understanding of DA development and PD pathogenesis should converge into rational therapeutic approaches with functional cell replacement being part of the strategy. Despite very little evidence that intrinsic neurogenesis plays a major role in PD pathology and possible repair, further understanding of the brain's capacity to restore function could advance this process. SC provide great opportunities, not only as a cell source, but also to model DA development and demise. While adult neural SC have limited differentiation and proliferation potential that constrains their application as a cell source of midbrain-like DA neurons, they have been shown to exert beneficial effects that may alter the progression of a number of neurodegenerative disorders. Unlike adult SC, pluripotent SC do not appear to have restrictions on their ability to differentiate into any somatic cell type, including midbrain DA neurons. Inductive protocols and coculture systems have been optimized to generate DA neurons that have the molecular signature and functional properties of midbrain SNC neurons. However, pluripotency is a "double-edge sword" and much work needs to be done to meet safety requirements without compromising efficacy for biomedical applications.

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 25

Gene Therapy for Parkinson's Disease

Takao Yasuhara and Isao Date

Abstract Parkinson's disease is characterized by the degeneration of the nigrostriatal dopaminergic neurons with the manifestation of tremor, rigidity, akinesia, and disturbances of postural reflexes. Medication using L-DOPA and surgeries including deep brain stimulation are the established therapies for Parkinson's disease. Cell therapies are also effective and have rapidly developed with the recent advancement in molecular biological technology including gene transfer. In this review, *ex vivo* gene therapy using genetically engineered cell transplantation for Parkinson's disease model of animals is described, including catecholamine/neurotrophic factor-secreting cell transplantation with or without encapsulation, as well as *in vivo* gene therapy using direct injection of viral vector to increase dopamine-production, ameliorate the survival of dopaminergic neurons, correct the deteriorated microenvironment, or normalize genetic abnormality. Furthermore, the future directions for clinical application are described together with recent clinical trials of gene therapy.

Keywords Cell therapy • Dopaminergic neurons • Encapsulation • Neural stem cell • Neurotrophic factor • Viral vector

Abbreviations

6-OHDA	6-hydroxydopamine
AADC	Aromatic-L-amino-acid decarboxylase
AAV	Adeno-associated virus
BHK	Baby hamster kidney cell
CNS	Central nervous system
Dox	Doxycycline
GAD	Glutamic acid decarboxylase
HSV	Herpes simplex virus

LRRK2	Leucine-rich repeat kinase 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSCs	Marrow stromal cells
PD	Parkinson's disease
STN	Subthalamic nucleus
SVZ	Subventricular zone
TH	Tyrosine-hydroxylase
UCH-L1	Ubiquitin C-terminal hydrolase L1
VEGF	Vascular endothelial growth factor
X-SCID	X-linked severe combined immunodeficiency

Introduction

Fifteen years ago, the development of genetic engineering altered the therapeutic strategies for some diseases, including adenosine deaminase deficiency (Bordignon et al. 1993). Recent reports of the development of leukemia in 3 X-linked severe combined immunodeficiency (X-SCID) patients treated in France inevitably affected the continuing development of research in this area (Hacein-Bey-Abina et al. 2003). After the occurrence, minimization of the risk to target the potentially pathogenic locus has been actively discussed and gene therapy is still progressing (Gonin et al. 2005). For diseases in the central nervous system (CNS), Aebischer reported encapsulated CNTF-producing cells for amyotrophic lateral sclerosis patients (Aebischer et al. 1996). Advancement of biotechnology in the recent decade, as typified by stem cell engineering, extended the dimension of gene therapy applicable to CNS diseases.

Parkinson's disease (PD) is a neurodegenerative disorder in the CNS, characterized by the dopaminergic neuronal loss in the nigrostriatal system with clinical manifestation as resting tremor, rigidity, akinesia, and disturbances of postural reflex. Initially, dopamine replacement therapy was established and is still a central pillar of the treatment of PD (Sethi 2002). Stereotaxic surgery, such as deep brain stimulation and electrical coagulation was also established

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as a solid therapeutic option for PD (Benabid et al. 2000; Vitek et al. 2003). On the other hand, cell transplantation (Lindvall et al. 1988) and continuous administration of glial cell line-derived neurotrophic factor (Gill et al. 2003) were initially considered as treatment of choice for PD patients. The therapies are still considered effective, although randomized controlled trials demonstrated limited significant therapeutic effects of fetal nigral cell transplantation for PD patients (Freed et al. 2001; Olanow et al. 2003) and also limited efficacy and safety of GDNF therapy for PD patients (Lang et al. 2006). Gene therapy for PD patient was started in the new millennium, although basic researches on gene therapy for PD were numerous (Wolff et al. 1989). Recent developments in PD treatment, suggest that gene therapy may provide a ray of hope.

The methods of gene therapy were divided into *in vivo* and *ex vivo* gene transfer. *In vivo* method is the direct gene delivery to a living body, including the direct injection of viral vector encoding neurotrophic factor for neuroprotective effects or enzymes to increase dopamine production. With *in vivo* methods, strong efficacy is expected despite relatively high risks of viral infection. Historically, there are many well-designed studies using *in vivo* methods with lentivirus (Georgievska et al. 2004), adeno-associated virus (Muramatsu et al. 2002) or Herpes simplex virus (Natsume et al. 2001) for treating PD. Further advances in genetic engineering might expand the possibility of *in vivo* gene therapy for PD. *Ex vivo* methods, on the other hand, involve transferring the gene of interest into cultured cells, then subsequently transplanting the transfected cells. Compared with *in vivo* gene therapy, the *ex vivo* approach poses relatively low risks to host brain cells. In both gene delivery methods, advantages and disadvantages of each approach need to be carefully considered and confirmation of safety is a prerequisite for proceeding with clinical application. In this review, *ex vivo* gene therapy is described with findings from the studies in our laboratory. Then ongoing *in vivo* gene therapies for PD are discussed, together with future developments.

Ex vivo Gene Therapy

Ex vivo gene therapy has specific advantages compared with other delivery systems (Behrstock and Svendsen 2004), including the following: viral transfection to cells is performed in the laboratory, thus reducing the risks of using live replication-competent virus transfer to the patients directly; promising cells with appropriate secretion of specific factors can be selected before transplantation; and viable and appropriate cells are usable by tailor-made

engineering instead of targeting degenerating host cells, which are likely to display low viability. With these advantages in mind, we describe here two different *ex vivo* methods generated in our laboratory.

Encapsulated Cell Transplantation

Our group has pursued the investigation of encapsulated cell transplantation for treating CNS diseases, including PD and cerebral ischemia (Date et al. 2000, 2001; Fujiwara et al. 2001; Shingo et al. 2002; Yoshida et al. 2003, 2004b, 2005b, Yano et al. 2005). Encapsulated cell transplantation has the following characteristics. First, various neurotransmitters, neurotrophic factors, or growth factors can be produced continuously from encapsulated cells with engineered properties. Cells inside the capsule are supplied with sufficient nutrient and oxygen through the semipermeable membrane. Second, the capsule can be removed from the transplanted brain if need be. Third, scant immune reactions and no immunological rejection arise, because cells inside are protected by the capsule. Additionally, there is no risk of tumorigenesis in the host tissue. Fourth, various cells including immortalized cell lines can be transplanted safely as a surviving donor with no ethical problems. These cells are also engineered genetically with ease. Fifth, encapsulated cell transplantation enables us to deliver continuous, low-dose administration of secreting factors to the surrounding host brain, even in the case that the half life of the secreted molecule is extremely short.

Catecholamine-Secreting Cell Transplantation

Initially, catecholamine administration using encapsulated cell transplantation was performed in our laboratory. To increase the local concentration of catecholamine in dopamine-depleted striatum, PC12 cells derived from rat pheochromocytoma with catecholamine-secretory potencies were encapsulated and transplanted into the striatum of a PD model of monkeys (Yoshida et al. 2003). Behavioral amelioration of monkeys with the encapsulated cell transplantation was demonstrated for 1 year with consequent scant immunological reaction around the graft and survival of cells inside the capsule by immunohistological investigations. Thus, local supply of catecholamine by encapsulated cell transplantation might be effective and feasible. We also confirmed the enhanced neuroprotective effects by tyrosine-hydroxylase (TH)-overexpressed PC12 cells in a PD model of rodents (unpublished data). Similarly, the TH gene was transduced into marrow stromal cells (MSCs) by AAV in other institutes (Lu et al. 2005). TH-MSCs were transplanted

into the striatum of a PD model of rats without encapsulation. Consequently, treated rats were behaviorally ameliorated with increased TH-immunoreactivity and dopamine-concentration by the transplantation.

Meanwhile, oversupply of dopamine for an extended period might make the pathological conditions of PD patients more complicated and sometimes lead to deterioration. Patients with catecholamine for extended periods often develop both dyskinesia and wearing-off (Nutt et al. 2002) with impairment of the synaptic dopamine metabolism in the putamen (Rajput et al. 2004). Additionally, two randomized controlled trials using fetal nigral cell transplantation clarified that cell transplantation-induced dyskinesia was recognized in some advanced PD patients, in spite of the established therapeutic effects (Freed et al. 2001; Olanow et al. 2003). Further PET study revealed that ^{18}F fluorodopa uptake in the putamen significantly increased in patients with dyskinesia (Ma et al. 2002), indicating the involvement of overdosed catecholamine administration in the dyskinesia. Consequently, we controlled catecholamine-secretion from the outside to achieve the ideal catecholamine dose, to reduce the side effects induced by overdosed catecholamine, and to enjoy the increased safety of cell transplantation. Specifically, using Tet-Off system, it was attempted to control catecholamine-secretion by oral doxycycline (Dox) administration. PC12 cell line (PC12TH Tet-Off) was established, in which human TH expression can be negatively controlled by Dox administration. The amount of secreted catecholamine from the cell line was confirmed to decrease by Dox administration in a dose responsive manner. Reverse transcription-polymerase chain reaction also revealed that the expression of human TH was clearly reduced by Dox administration. In addition, the changeover of catecholamine secretion by Dox administration was found for 70 days *in vivo*, although the control became less effective with time. Subsequently, encapsulated cells were transplanted to the striatum of a PD model of rats. Behavioral amelioration was achieved by catecholamine-secretion from the capsule. Adversely, behavioral deterioration was found by Dox administration. The catecholamine concentration in the CSF was also controlled by Dox administration with long-term survival of encapsulated cells, although these effects gradually reduced *in vivo* (Kobayashi et al. 2006).

Neurotrophic Factor-Secreting Cell Transplantation

GDNF is a neurotrophic factor with potent neuroprotective effects on dopaminergic neurons (Lin et al. 1993). In parallel with catecholamine-secreting encapsulated cell transplantation, GDNF therapy was also explored using a PD model of rodents in our laboratory, as summarized in the recent review by Lindvall (Aoi et al. 2000; Date et al. 2001; Shingo

et al. 2002; Yasuhara et al. 2005b; Lindvall and Wahlberg 2008). After confirmation of the neuroprotective and neurorestorative effects of GDNF through GDNF receptor $\alpha 1$, we then explored the therapeutic potencies of a well-known angiogenic factor, vascular endothelial growth factor (VEGF). At that time, multiple roles of VEGF other than angiogenesis, that is, neuroprotective or neurogenic potencies were recognized and highlighted (Yasuhara et al. 2004a). There were a few studies on the therapeutic effects of VEGF. Silverman and colleagues demonstrated neuroprotective effects of VEGF on rat mesencephalic explant cultures through the indirect mechanisms representing angiogenesis and glial proliferation (Silverman et al. 1999). Following this study, neuroprotective effects of VEGF on cultured dopaminergic neurons were confirmed. Then, we proceeded to encapsulated VEGF-secreting cell transplantation for a PD model of rats. VEGF-secreting cell line (baby hamster kidney cell (BHK)-VEGF) was made using a cationic liposome-mediated DNA delivery system. One million cells were encapsulated into polymer hollow fibers, with consequent secretion of 10 ng per day for at least 6 months. The encapsulated cells were unilaterally implanted into the striatum of adult rats before the administration of 6-hydroxydopamine (6-OHDA) into the affected striatum. The rats receiving the BHK-VEGF capsule showed a significant decrease in rotational behavior, significant preservation of TH positive neurons in the substantia nigra pars compacta, and fibers in the striatum, compared with the control group. In addition, angiogenesis and glial proliferation were recognized around the capsule. The neuroprotection might be mediated by both indirect and direct mechanisms through VEGF receptor2 (Yasuhara et al. 2004b). Subsequently, the therapeutic dose window was explored using different sizes of capsules with different amount of VEGF secretion (Yasuhara et al. 2005c). This study suggests that continuous administration of low-dose VEGF is essential for neuroprotection, although high-dose VEGF induced severe brain edema with subsequent decreased neuroprotective effects, thus suggesting the narrow therapeutic dose window. This finding was also verified by several groups (Manoonkitiwongsa et al. 2004; Ozawa et al. 2004). Other groups also reported that intraventricular administration of low-dose VEGF exerted neurorescue effects on a stroke model of mice, but not intravenous administration of high-dose VEGF (Kaya et al. 2005). Furthermore, neurorestorative effects of VEGF upon damaged dopaminergic neurons were demonstrated when VEGF was administered after 6-OHDA lesion both *in vitro* and *in vivo* (Yasuhara et al. 2005a). VEGF has strong effects upon endothelial cells, glial cells, and neurons, thus suggesting that the possible potencies for clinical application in the brain, as ischemic limb or heart is a good target for VEGF in clinical practice (Mohler et al. 2003; Reilly et al. 2005).

However, VEGF might be a double-edged sword, especially when used in the brain, and therefore future preclinical studies should proceed with caution.

Further Application of Encapsulation Technique for Stem Cell Research

Thus, the transplantation of encapsulated cells holds great promise for clinical applications in various CNS diseases. Additionally, encapsulation technique might contribute to the revealing of the mechanisms of therapeutic effects using stem cells. There is little evidence of cell replacement of damaged cells, as well as reinnervation of the host CNS with stem cell transplantation. Moreover, the ratio of neuronal differentiation of stem cells derived from a nonneuronal source is very limited (Chu et al. 2004). Despite the minimal neuronal differentiation and host reinnervation, the occurrence of functional recovery in a transplanted PD model of animals suggest that secretion of humoral factors by stem cells is a more plausible explanation for such robust neuroprotection (Ourednik et al. 2002). Neural stem cells are also reported to secrete neurotrophic factors, including pleiotrophin, GDNF, and stem cell factor, suggesting that these factors exert neuroprotection and promote both differentiation to dopaminergic neurons and extensive host axonal growth after spinal cord injury (Lu et al. 2003; Chu et al. 2004; Yasuhara et al. 2006). Neurotrophic factors can be secreted from the capsule, although there is no reinnervation of the host achieved with encapsulated cell transplantation. A thorough comparison of stem cells with and without encapsulation might shed crucial light on the specific biology of transplanted stem cells. Furthermore, selective differentiation of neural progenitor cells has been achieved by high-epitope density nanofibers (Silva et al. 2004), thus suggesting the potential of using the capsule as a scaffold for neuronal differentiation.

Neurotrophic Factor-Secreting Stem Cell Transplantation

Various stem cells are known to have potencies to self-renew, to migrate where they are required, and to differentiate into various lineages, such as neurons and glial cells. Kurozumi and colleagues demonstrated that mesenchymal stem cells from bone marrow, genetically engineered to secrete neurotrophic factors, were transplanted into the striatum of a middle cerebral artery occlusion (MCAO) model of rats and subsequently displayed neurorescue effects (Kurozumi et al. 2004). The ability of MSCs to migrate

widely was of a great advantage to disperse neurotrophic factors into the required area. A similar method using neural stem cells was attempted in a PD model of rats in our laboratory, because immature stem cells can supply neurotrophic factor or growth factor as well as reinnervation with host tissues. To enhance the function of factor resource, genetically engineered adult neural stem cells were transplanted into a PD rat model. Adult neural stem cells were isolated from the subventricular zone (SVZ) where neural stem cells with continuous neurogenesis resided. Neural stem cells were cultured using the neurosphere technique with epidermal growth factor for a few weeks, then transfected using fiber-mutant F/RGD adenovirus vector containing the GDNF gene (Kameda et al. 2007). The resulting cells with sufficient secretion of GDNF were transplanted into PD model of rats. As a result, the transplanted rats showed behavioral improvement over time. Furthermore, the immunohistochemical investigation demonstrated many surviving transplanted cells and preserved TH-positive fibers in the striatum and neurons in the substantia nigra pars compacta (Muraoka et al. 2008). Similarly, GDNF- or neurotrophin-3-secreting neural stem cells were used for a PD model of animals as minipumps to release GDNF/neurotrophin-3 *in vivo* to protect aging dopaminergic neurons (Lu et al. 2003; Behrstock and Svendsen 2004).

Nurr1 Overexpressed NT2N Cell Transplantation

Recently, Borlongan and colleagues showed interesting data, using the human embryonic carcinoma NT2 cell line carrying the human Nurr1 gene (NT2N.Nurr1) (Hara et al. 2008). Cells derived from tumor-like NT2 cells or embryonic stem cells are easy to increase, as well as to differentiate into specific lineages and are considered as good candidates for cell therapy. However, this highly potent proliferative property should be controlled completely before clinical application. Postmitotic status of NT2N cells induced by treatment with retinoic acid and mitotic inhibitors is considered as relatively safe and is clinically used for stroke patients (Kondziolka et al. 2000). By transfecting Nurr1 using the retrovirus, an orphan nuclear receptor, critical to dopamine expression during early development and maintenance of the dopaminergic neurons in the midbrain, Borlongan established NT2N.Nurr1 cells with both a promoted neuronal commitment and a secretory function of GDNF. NT2N.Nurr1 transplantation for a stroke model of animals ameliorated the behavioral impairment with the reduction of histological damage, demonstrating the mighty therapeutic potencies of the cells (Hara et al. 2007). Next we proceeded to the application of NT2N.Nurr1 cells for a

PD model of rodents. Differentiated NT2N.Nurr1 cell transplantation ameliorated behavior and histology in a PD model of rats induced by 6-OHDA lesion (unpublished observation)

In vivo Gene Therapy

The prominent characteristics of *in vivo* gene therapy are demonstrated in direct gene delivery using vectors with subsequent potent transfection and therapeutic effects, in spite of a relatively high risk of viral infection. Herpes simplex virus (HSV), Retrovirus, adenovirus, adeno-associated virus (AAV), lentivirus, and other nonviral vectors like liposome are usable for gene therapy, although they have differing advantages and disadvantages. Retrovirus is a RNA virus, characterized with a moderate efficiency of transduction to various dividing cells. The genome is randomly incorporated into the cells with a low expression level of transfected gene. Adenovirus is a DNA virus, characterized by a very high efficiency of transduction to both dividing and nondividing cells, in spite of the toxicity and transient gene expression. Using AAV, transduction to both dividing and nondividing cells is also possible with relatively stable expression for a long time without pathogenicity. Lentivirus is also hopeful with the stable expression in dividing and nondividing cells. Nonviral vector might be safe, although the low transduction rate and transient expression should be improved. Historically, for the CNS diseases, HSV was used initially for malignant tumors (Packer et al. 2000). For safety, a regulatable system of gene expression of the vector is promising, although a further technical breakthrough is awaited (Cress 2008). Björklund predicted the clinical application of AAV or lentivirus to supply GDNF in degenerated dopaminergic systems. At present, biotechnology using viruses is steadily being developed. Three clinical trials of gene therapies are ongoing for PD using AAV (Kordower and Olanow 2008). In this section, the strategies of gene therapy for PD are discussed. Subsequently, recent gene therapy using AAV is highlighted, although other viral vectors might also be good tools for PD treatment (Natsume et al. 2001).

The Strategies of In vivo Gene Therapy for PD

With *in vivo* methods, the strategies for PD might be roughly classified into four groups: increase of local dopamine concentration; neuroprotection and neurorestoration for degenerated dopaminergic neurons; amelioration in the microenvironment of dopaminergic/nondopaminergic systems involved in PD,

and normalization of genetically abnormal cells related to the pathogenesis of PD.

Since PD is characterized by dopaminergic degeneration, and catecholamine replacement is effective, the attempt to increase dopamine production is indicated. Kirik used AAV vector to transfect TH and GTP-cyclohydrolase, a cofactor synthetic enzyme into the striatum of a PD model of rats with subsequent behavioral amelioration (Kirik et al. 2002). Muramatsu injected the mixtures of three separate AAV vectors expressing TH, aromatic-L-amino-acid decarboxylase (AADC), and GTP-cyclohydrolase into the putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys. Consequently, the manual dexterity and TH immunoreactivity were ameliorated as dopamine concentration increased (Muramatsu et al. 2002).

The pathogenesis of PD is gradually being discovered, but is as yet not fully known. However, radicals, mitochondrial dysfunction, and apoptosis are known to be involved in the sideration of PD (Barzilai et al. 2000; Barzilai and Melamed 2003). GDNF exerts neuroprotection and neurorestoration (Aoi et al. 2000). Several groups induced GDNF into the nigral dopaminergic neurons with successful therapeutic effects (Ozawa et al. 2000 Wang et al. 2002; Kordower 2003). Other than GDNF, Bcl-2 or neurturin was used similarly (Natsume et al. 2001; Marks et al. 2008).

In PD patients, the subthalamic nucleus (STN) and globus pallidus pars interna were hyperactivated in a vicious circle (Yasuhara and Date 2007), thus demonstrating an electrophysiologically exacerbated microenvironment in PD. Deep brain stimulation for the STN was believed to suppress the hyperactivity of the STN with drastic amelioration of PD symptoms by normalization of the electrophysiological circuit (Benabid et al. 2000). During has developed AAV-glutamic acid decarboxylase (GAD) injection into the STN and has achieved the suppressive effects on the hyperactivated STN, which is similar to the underlying mechanism of DBS (During et al. 2001). Other gene therapy aiming at the amelioration of the microenvironment includes dopamine-receptor upregulation. In PD patients, D2/D3 agonist exerts neuroprotection (Linazasoro et al. 2008). Borlongan and colleagues overexpressed D2/D3 dopamine receptor genes in the dopamine-denervated striatum of rodents, using lentiviral vector and behaviorally demonstrated the enhanced therapeutic effects of ropinirole, a D2/D3 agonist with the involvement of enkephalin and substance P immunoreactive medium spiny neurons (Matsukawa et al. 2007). Thus, the amelioration of the microenvironment might be a good strategy for PD.

Several genes might be linked to the pathogenesis of PD, such as α -synuclein, leucine-rich repeat kinase 2 (*LRRK2*), and ubiquitin C-terminal hydrolase L1 (*UCH-L1*) (Gasser 2007; Belin and Westerlund 2008). Recent developments in genetic manipulation including dominant negative or

siRNA enables us to suppress the target gene expression, as well as conventional way of overexpression of the target genes (Porras and Bezard 2008). Additionally, a PD model of animals by genetic manipulation of nigral cells might be useful to understand the pathogenesis of PD (Ulusoy et al. 2008). In the future, the enhancement or suppression of the target gene might be a strong tool for some types of PD.

Ongoing 3 Clinical Trials of Gene Therapy for PD Using AAV

Perhaps coincidentally, three clinical trials using AAV began in 2006, although each strategy is independent of the others (Kaplitt et al. 2007; Eberling et al. 2008; Marks et al. 2008; Kordower and Olanow 2008). Two approaches are directed toward symptomatic benefit, while one is modeled for both disease modifying and symptomatic effects (Cress 2008). Such a 'coincidence' may be due to the special advantages of AAV vectors. AAV are considered as promising gene-delivery vehicles, because they are derived from a nonpathogenic virus with highly efficient transduction into nondividing cells and consequent relatively stable gene expression for an extended period. AAV viral capsid might induce an immune reaction and the neutralizing antibodies to AAV viral capsid might reduce the efficiency of viral transduction, with subsequent therapeutic effects. In several serotypes, AAV2 vector is used for clinical trials, because it is suitable for neuron-specific transfection. On the other hand, AAV5 provides efficient transduction (Ozawa et al. 2000; Ozawa 2007).

Intrastriatal AAV-AADC infusion for a PD model of monkeys demonstrated that low doses of L-dopa were converted to dopamine in the affected striatal neurons with ameliorated behavior with no side effects. After a preclinical study, a phase I safety trial was initiated using AAV-AADC. Five advanced PD patients received a bilateral infusion of a low-dose AAV-AADC vector into the putamen. PET scans demonstrated a 30% increase of AADC tracer in the putamen. Additionally, the safety of the involved patients might be improved with the amelioration of the symptoms without drug-induced dyskinesia, although a randomized controlled trial might be required (Eberling et al. 2008).

As described in the previous section, the delivery of GAD gene to the STN decreases the hyperactivity of the nucleus similar to DBS (During et al. 2001). This study has also reached phase I clinical trials with no adverse events related to gene therapy. Significant improvements in motor UPDRS scores were demonstrated 12 months after gene therapy (Kaplitt et al. 2007). FDG-PET study demonstrated correlation with the abnormal metabolism usually seen in PD patients (Feigin et al. 2007). Both of the gene therapy approaches

might be symptomatic and are not involved in the progression of PD (Mochizuki et al. 2008) with subsequent stepup to phase II trials.

To achieve neuroprotection and neurorestoration for PD patients, a phase I trial using AAV2-neurturin was started after the demonstration of therapeutic effects in a preclinical study using rodents and nonhuman primates (Gasmi et al. 2007; Herzog et al. 2007). CERE-120, an AAV2-based gene delivery vector encoding neurturin, was advanced to a phase I clinical trial. No side effects were recognized for 1 year with a significant amelioration of motor function with an increased on-time without troublesome dyskinesia (Marks et al. 2008).

Perspectives

In coming years, phase II randomized controlled trial of *in vivo* gene therapy will be developed; it is hoped for with therapeutic effects, by the increase of dopamine production, correction of electrical circuits, or neurorestoration of affected dopamine neurons. Further preclinical safety tests might finally lead to clinical trials using *ex vivo* methods. As described in the *in vivo* gene therapy section, gene therapy might achieve symptomatic relief or slow disease progression in accordance with target genes.

Maguire-Zeiss usefully suggested the identification of novel targets that are amenable to early intervention, prior to the substantial loss of dopamine neurons. Today, PD patients are diagnosed mainly by clinical evaluation, because there is as yet no supporting evidence of certified biomarkers, although recent studies have demonstrated promising molecules (Yasuhara et al. 2007; Gerlach et al. 2008). As is widely understood, the degeneration of dopaminergic neurons is progressed when representative symptoms, such as resting tremor, rigidity, and akinesia appear. In the near future, PD patients will be diagnosed very early in the course of disease prior to the appearance of any clinical symptoms in motor function, by a combination of biomarker analyses and clinical evaluations of nonmotoric symptoms. Hopefully, advanced imaging tools will help to target and monitor treatment. Early intervention might include exercise and environmental enrichment as well as antiinflammatory, neurotrophic, and antioxidant agents. The agents might be delivered pharmacologically or perhaps in cases of long-term treatment, via gene therapeutic approaches (Maguire-Zeiss et al. 2008). With early intervention, gene therapy might alter dramatically the course of PD and consequently the lives of PD patients (Fiandaca et al. 2008).

Conflicts of interest statement We declare that we have no conflict of interest.

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Chapter 26

Immunization as Treatment for Parkinson's Disease

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Abstract Parkinson's disease and other neurodegenerative disorders share a common pathologic pathway with aggregation and deposition of misfolded proteins causing a disruption of particular neuronal networks. Several mechanisms have been implicated in the downstream events following deposition of misfolded proteins including free radical formation and failure of cellular defences such as autophagy or protein-degradation by the ubiquitin-proteasome pathway among many others. Treatments, however, capable of arresting or at least effectively modifying the course of disease do not yet exist. Recently, immunization approaches including passive and active immunization have been tested in animal models of various neurodegenerative disorders and have already entered into clinical trials for the treatment of Alzheimer's disease. In this review, we specifically focus on the current status of immune-based approaches that are presently developed as a potential therapy of Parkinson's disease.

Keywords Parkinson's disease • Alpha-synuclein • Immunization • Neuroprotection

Abbreviations

A β	beta-Amyloid
CFA	Complete Freund's Adjuvant
GDNF	Glial Cell-line derived Neurotrophic Factor
GFAP	Glial fibrillary acidic protein
HE Stain	Haematoxylin and Eosin Stain
Iba 1	Ionized calcium-binding adaptor molecule 1
IHC	Immunohistochemistry
LB	Lewy Body
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
PD	Parkinson's disease

scFv	Single chain variable Fragment
ThT	Thioflavin T
UCH-L1	Ubiquitin Carboxyl-terminal Esterase L1

Introduction

Among neurodegenerative diseases, idiopathic Parkinson's disease (PD) is only exceeded by Alzheimer's disease. The prevalence varies from 0.3% in the general US population (Weintraub et al. 2008) to >5% in individuals >80 years (von Campenhausen et al. 2005). PD is clinically characterized by its motor symptoms including bradykinesia, resting tremor, rigidity, and later in the disease, postural instability. The pathophysiological correlate of the motor symptoms is a prominent cell loss (>60% in the ventral tier of the pars compacta) of melanin containing dopaminergic neurons in the substantia nigra. This loss leads to a disruption in pallidal inhibitory influences of the subthalamic excitatory system in the basal ganglia, with a subsequent pronounced inhibition of the motor control in the brain stem, thalamus, and consequently the cortex (Lang and Lozano 1998). Several mechanisms and factors were proposed as a cause of the selective neurodegeneration: oxidative stress was one of the first mechanisms considered to be responsible for cell death because of the ability of dopamine to produce quinones, peroxides, hydroxyl radicals, and other reactive oxygen species (Lang and Lozano 1998; Olanow 2007). Excitotoxicity was also proposed as a factor because of an increase in free calcium due to glutamate mediation after dopamine depletion (Olanow 2007). Furthermore, mitochondrial dysfunction was accused to cause cell death, either by defects in complex I in the respiratory chain or by apoptosis induction via BAX, cytochrome *c* release and subsequent caspase activation (Gandhi and Wood 2005; Olanow 2007). Finally, most familial forms of PD are caused by mutations in genes involved in protein processing: UCH-L1 (Park5) and Parkin (Park2) are ubiquitin ligases and proteases, relevant in the

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ubiquitin proteasome system for protein degradation (Gandhi and Wood 2005; Olanow 2007). This etiopathophysiology is most clearly demonstrated by the first genetic mutation (PARK1) described in the α -synuclein gene (Polymeropoulos et al. 1997). α -synuclein, an unfolded, monomeric 140 amino acid protein, is usually present in presynaptic terminals. Here it was reckoned to exert some effects such as activity-dependent membrane channels (in dopamine release), synaptic plasticity, or chaperone-like abilities similar to the 14-3-3 protein. In PD, α -synuclein tends to misfold and/or to aggregate if present in mutated forms or in excess (Tofaris and Spillantini 2007) and is one of the major components of Lewy neurites and Lewy bodies, one histopathological hallmark of PD (Spillantini et al. 1997). Classical Lewy bodies are spherical, intraneuronal, intracytoplasmatic inclusion bodies 8–30 μ m in size. α -synuclein seems to play a key role during Lewy body formation as deposits can be detected throughout the different stages: Stage 1: diffuse, pale, cytoplasmic immunostain in otherwise normal appearing neurons; Stage 2: irregular, uneven, and moderate staining in often poorly pigmented neurons; and Stage 3: discrete cytoplasmatic immunostaining corresponding pale bodies in an haematoxylin and eosin (HE) stain (well defined, less eosinophilic (“pale”) glassy areas without halo in pigmented neurons). These pale bodies often display peripheral condensations or small classical Lewy bodies composed of core and halo. Stage 4: typical immunostain of a classical Lewy body showing a strong stained halo around a weak core.

Until a few years ago, most researchers considered the Lewy body to be a toxic agent that is responsible for neurodegeneration: now a common view is that the Lewy body is an aggresome that segregates potentially toxic components (oligomers of α -synuclein) from neuronal compartments. (Olanow et al. 2004; Wakabayashi et al. 2007). Therefore, the Lewy body and its prominent component α -synuclein serve as a rational target for therapeutic approaches. Recently, different immunization-based approaches were developed to specifically target this protein, which are the focus of this review.

The Development of Immunization in Neurodegenerative Disorders

Since the end of the eighteenth century, immunization has become a common therapeutic approach against infectious disease. Usually an antigen is applied to initiate an immune response with endogenous antibody production (so-called “active immunization”), while the administration of exogenous antibodies is used as passive immunization. Passive immunization is now applied in a broad spectrum of diseases such as autoimmune diseases (Delavallee et al. 2008) and

cancer (Baxevas et al. 2008), and over the last decade, immunization approaches have also been developed for the treatment of neurodegenerative diseases (Brody and Holtzman 2008). In the first ground-breaking paper, Schenk and co-workers demonstrated in a transgenic mouse model (PDAPP) that active immunization directed against beta-Amyloid ($A\beta$) reduced the plaque load considerably (Schenk et al. 1999). Similar results were replicated with passive immunization, different applications, and other transgenic mouse models (Bard et al. 2000; Lemere et al. 2000; DeMattos et al. 2001). Supportive data have shown that passive immunization with antibodies directed against amyloid also ameliorates behavioral deterioration and may even clear existing plaques (Bard et al. 2000; DeMattos et al. 2001). Based on those results, a clinical trial using an active immunization approach with full-length $A\beta_{1-42}$ (and QS-21 as an immun adjuvant) was initiated as soon as 2001, but had to be stopped due to the development of acute meningoencephalitis (Gilman et al. 2005). Lately, several new preparations, capable of providing antibodies against $A\beta$ by either active or passive immunization, have been formulated and have reached clinical testing (for further review see (Solomon 2007; Brody and Holtzman 2008; Neff et al. 2008). Also passive and active immunization approaches were developed for other neurodegenerative disorders such as Prion diseases (Wisniewski and Sigurdsson 2007) and amyotrophic lateral sclerosis (Urushitani et al. 2007). None of the later approaches, however, has yet entered into a clinical trial.

Immunization as a Treatment for Parkinson’s Disease

Active Immunization

Masliah and colleagues reported that vaccination against α -synuclein could provide a new approach to the treatment of PD (Masliah et al. 2005). They were able to demonstrate that α -synuclein antibodies were generated with adequate titers in mice following immunization. Three and six month old human α -synuclein transgenic mice (Masliah et al. 2000) were immunized for 8 months with recombinant α -synuclein or complete Freund’s adjuvant (CFA). A considerable reduction of human α -synuclein deposits in the neuronal cell bodies and synapses and a preservation of synaptophysin-immunoreactive nerve terminals were detected in immunized animals. This effect was dependent on the antibody’s relative affinity. The inflammatory response, which was found following immunization with α -synuclein and QS-21 in AD, measured by microglia activation (via Iba1 IHC) and gliosis (GFAP) was mild and was not different between the

groups. However, the mechanisms by which the antibodies may recognize and promote clearance of the intracellular accumulation of α -synuclein are not well understood. It was proposed that antibodies may either recognize membrane-bound α -synuclein on the cell surface or interact with surface structures (receptor-dependent or receptor-independent) and are consecutively internalized into intracellular compartments. Those internalized antibodies may then promote degradation via the activation of lysosomal pathways. An immune mediated cellular response was excluded based on their results.

The authors concluded that vaccination is effective in reducing the neuronal accumulation of synuclein aggregates and may have a potential role in the treatment of synucleinopathies. Unfortunately, no further studies have been published using active immunization in models of PD and a large number of questions raised by this study remain unanswered.

Passive Immunization

Passive immunization for PD is a promising approach to avoid potential inflammatory response in the brain, which may be triggered by active immunization. First, Benner and colleagues reported vaccination with Copaxone-based regimens (Benner et al. 2004). They could demonstrate that copolymer-1 immune cells administered to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice suppress microglial responses and increase the expression of glial cell-line derived neurotrophic factor (GDNF). This therapeutic immunization strategy resulted in a significant protection of nigrostriatal neurons against MPTP-induced neurodegeneration that was abrogated by depletion of donor T-cells. Also, Messer and colleagues employed copolymer-1 to affect specific T-cell responses to control unwanted side effects of the immune response in an animal model (Miller and Messer 2005).

Emadi and co-workers characterized an antibody fragment against α -synuclein that inhibits the formation of protofibrils, which are thought to be the toxic species of α -synuclein (Emadi et al. 2004). By panning a phage display library against monomeric and oligomeric forms of α -synuclein, they could isolate a specific single-chain antibody fragment (scFv) that binds exclusively to dimeric or tetrameric forms of α -synuclein. In a cell culture model, these scFv were shown to reduce the toxicity of α -synuclein protofibrils. Zhou and colleagues similarly showed that an anti-monomeric α -synuclein scFv, preferentially binds to α -synuclein in a monomeric form rather than in the toxic oligomeric state (Zhou et al. 2004). Maguire-Zeiss and coworkers are taking the conformation issue further, since

the group is testing conformation specific anti α -synuclein scFvs that could help to determine the toxic α -synuclein species (Maguire-Zeiss et al. 2006). Similar results were also reported from extracellularly deposited A β in Alzheimer's disease (Games et al. 2000; Lemere et al. 2000; Morgan et al. 2000) and intracellular proteins in the case of Huntington's disease (Luthi-Carter 2003) as well as membrane associated molecules such as the prion protein (Bainbridge and Walker 2003).

Recently, Papachroni et al. investigated the prevalence of naturally occurring autoantibodies against α -, β -, and γ -synuclein in patients of familial and sporadic PD as well as in healthy controls, by incubation of the patients' sera with recombinant α -, β -, and γ -synuclein protein in Western blots (Papachroni et al. 2007). Autoantibodies against α -synuclein were present in 90% of sera of familial PD, in 51% of sporadic PD, but only in 31% of the control sera. There was no significant correlation of the antigenicity or antibody titres with disease-related factors like age of onset, stage of disease, exposure to pesticides, etc.

Furthermore, our group was able to purify those human α -synuclein antibodies (α -syn-Abs) from immunoglobulin G preparations (IVIg) and serum. We found that the resulting fractions had a strong anti α -synuclein signal compared with the flowthrough IgG as well as peripheral fractions without α -syn-Abs. The binding specificity of the α -syn-Abs could be confirmed with immunoprecipitation and surface plasmon resonance analysis. To determine the cellular and subcellular localization of the binding partners of the α -syn-Abs in the brain of patients with PD, immunohistochemical experiments were conducted. In PD patient histopathological samples, the α -syn-Ab recognizes the same structures as the monoclonal antihuman α -syn-Ab, e.g., as a halo around a weaker core in Lewy bodies and as drilled roots in Lewy neurites. Further investigations are necessary, regarding the physiologic effect of these naturally occurring autoantibodies on the neurotoxicity of α -synuclein.

Conclusion

Various methods for active or passive immunization are being pursued as potential therapies for several neurodegenerative diseases characterized by the accumulation of misfolded proteins (Brody and Holtzman 2008). Masliah and colleagues provided first evidence that this approach could also be applicable to synucleinopathies, including Parkinson's disease and dementia with Lewy bodies, in which insoluble forms of α -synuclein aggregate in characteristic cytoplasmic inclusions. Although limited to mouse models, the data currently available open up the possibility that anti α -synuclein antibodies may block or at least inhibit the

neurotoxic effects of excess α -synuclein by promoting the degradation and clearance of these aggregates. If applicable to humans, the immunization approach could provide a way to slow down the neurodegenerative process in patients with various forms of synucleinopathies, including Parkinson's disease. It remains to be seen whether passive or active immunization will be pursued in the field of PD. However, active immunization strategies in humans using endogenous proteins or protein derivatives undoubtedly have a higher risk of triggering adverse immune responses.

Conflicts of interest statement RD and MB hold patents on passive immunization in Alzheimer's disease. They have received grants and honoraria from various industrial companies for research.

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Chapter 27

A Diet for Dopaminergic Neurons?

Giuseppe Di Giovanni

Abstract Parkinson's disease (PD) is the second most common neurodegenerative disease, which unfortunately is still fatal. Since the discovery of dopamine (DA) neuronal cell loss within the substantia nigra in PD, the past decades have seen the understanding of the pathophysiological mechanisms underlying the degenerative process advance at a very impressive rate. Nevertheless, there is at present no cure for PD. Although there are no proven therapies for prevention, a large body of evidence from animal studies has highlighted the paramount role of dietary factors in counteracting DA degeneration. Consistently, associations between the risk of developing PD and the intake of nutrients, individual foods, and dietary patterns have been recently shown. Therefore, promoting healthy lifestyle choices such as a Mediterranean diet might be the key to reducing the risk of PD.

Keywords Dietary recommendations • Dopaminergic neurons • Neurodegeneration • Neuroprotection • Parkinson's disease • Prevention

Abbreviations

%CoQ10	Percentage of oxidized/total CoQ10
6-OHDA	6-hydroxydopamine
AD	Alzheimer disease
BMI	Body Mass Index
CoQ10	Coenzyme Q10
CSF	cerebrospinal fluid
DA	Dopamine
DATATOP-study	Deprenyl and Alpha-Tocopherol Anti-oxidative Therapy of Parkinsonism
DHA	Docosahexaenoic acid
DOPAC	3,4-dihydroxyphenylacetic acid

EGCG	(-)-epigallocatechin-3-gallate
GSH	Glutathione
HD	Huntington's disease
HVA	Homovanillic acid
KGDH	Ketoglutarate dehydrogenase
LDL	Low-density lipoprotein
MPP ⁺	1-methyl-4-phenyl pyridium
MPTP	N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MUFAs	Monounsaturated fatty acids
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAQUID	Personnes Agees Quid
PD	Parkinson's disease
PINK1	PTEN induced putative kinase 1
PUFAs	Polyunsaturated fatty acids
SNc	Substantia nigra pars compacta
SOD	Superoxide dismutase
TH	Tyrosine hydroxylase
TH-ir	Tyrosine hydroxylase immunoreactive
USDA	United States Department of Agriculture
Vitamin C	Ascorbic acid
Vitamin E	α -tocopherol

Introduction

Diet and nutrition are recognized cornerstones in the promotion and maintenance of good health throughout the entire life course. Their role as determinants of chronic diseases such as obesity, diabetes mellitus, cardiovascular disease, hypertension, stroke and some types of cancer is well established and they therefore occupy a prominent position in prevention activities (Willett 2008). Indeed, nutrition is showing itself to be a major modifiable determinant of chronic disease, with scientific evidence increasingly supporting the view that alterations in diet affect health throughout life. Indeed, dietary adjustments may not only influence present health, but may also determine whether or not an

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individual will develop chronic diseases much later in life (World Health Organ 2003).

Only now we are beginning to learn about how diet can impact on mental well-being and brain-aging and about the many nutrients that are now believed to be involved (Bourre 2006a,b). Recently, the importance of diet and specific dietary components in modifying the risk of developing neurodegenerative disorders, including Alzheimer's, Parkinson's and Huntington's disease (AD, PD, and HD), has been receiving a great deal of attention (Lau et al. 2007). Although each of these disorders may have a different principal cause, they share some cellular and molecular abnormalities that are believed to be pivotal to the dysfunction and ultimate death of the neurons. The abnormalities include a large number of factors such as genetic vulnerability, exogenous or endogenous toxins, hydroxyl radicals production, neuronal metabolic disturbances, disruption of cellular calcium homeostasis, inflammation, and apoptosis (Di Giovanni 2007, 2008; Hirsch et al. 1998; Sian et al. 1999; Jellinger 2000; Gebicke-Haerter 2001; Hartmann and Hirsch 2001; Jenner and Olanow 2006; Novikova et al. 2006).

Diet may play both a causative and preventive role in the etiology of these neurodegenerative disorders, for example, by effecting the neuronal membrane constitution, altering the oxidative balance in the brain, or serving as a vehicle for environmental neurotoxins. Although more basic research may be needed on some aspects of the mechanisms that link diet to brain health and normal aging, the currently available scientific evidence provides a sufficiently strong and plausible basis to justify taking action now. Compelling clinical and epidemiological evidence suggests that lifestyle factors, especially nutrition, may be crucial in AD prevention (Burgener et al. 2008). Consistently, experimental dietary regimens may promote, attenuate, or even partially reverse features of AD (Wang et al. 2005; Pasinetti and Eberstein 2008).

As far as PD is concerned, few epidemiological studies have been able to examine potential associations of diet and the risk for developing this disease because of its relatively low incidence and insidious onset. Potential roles of foods and nutrients in determining PD risk have been investigated. However, the results for many of them are still elusive, since dietary factors are difficult to assess. The important role that foods play in modulating PD neurodegeneration offers a significant challenge and an opportunity for valuable research attempts in the future.

Diet and Parkinson's Disease

PD is the second most common neurodegenerative disease in the elderly population affecting more than 2% of the population above 65 years of age with an inevitable exitus (Di Giovanni 2008). Since the underlying mechanisms of

dopamine (DA) neuronal loss in patients are not known yet, current therapies are mainly symptomatic and do not halt the progression of the disease (Schapira 2005). Therefore, an urgent need exists to find a means of preventing, delaying the onset, or reversing the course of the disease (Di Giovanni 2008). Even delaying the onset by a few years would decrease significantly its prevalence and subsequent burden on public-health systems. It is estimated that close to 4 million people worldwide suffer from PD. Moreover, the number of PD sufferers is expected to grow as the general population in the Western world ages. In fact, it has been estimated that this number will double to between 8.7 and 9.3 million by 2030 (Dorsey et al. 2007).

Since the symptoms of PD do not appear until up to 80% of the DAergic nerve cells have been lost (Hornykiewics 1988; Yurek and Sladek 1990), it is tempting to believe that some disease-promoting factors may be influenced by life-habits (Alexi et al. 2000; Di Giovanni 2008). A major one of these is nutrition that might slow and halt DAergic neuronal degeneration even preventing the disease.

Energy Intake

Around the world people from developed and developing countries are facing an obesity epidemic. Overeating and obesity is a major risk factor for chronic diseases (World Health Organ 2003). Conversely, dietary restriction has wide-ranging health benefits and is known to prolong life-span extending the survival of diverse species from rodents (Weindruch et al. 1986) to humans (Roth et al. 2002). In addition, reducing calorie intake is also now considered very important for the development and prevention of neuronal loss in aging and neurodegenerative disorders (Martin et al. 2006; Prolla and Mattson 2001; Duan and Mattson 1999; Mattson 2003; Maswood et al. 2004).

As far as the SNc DAergic neuronal death is concerned, a reduction of food intake by 40–60% without malnutrition has recently been shown to protect these neurons from N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin in mice (Duan and Mattson 1999) and in monkeys (Maswood et al. 2004). Surprisingly, caloric restriction did not show any neuroprotective effect against the neurotoxin 6-hydroxydopamine (6-OHDA) in rats (Armentero et al. 2008). Nevertheless, it is more likely that this lack of effect is due instead to the short length of dietary restriction administered and the more pronounced 6-OHDA neurotoxic insult in this latter study (Armentero et al. 2008) compared with those obtained with MPTP (Duan and Mattson 1999; Maswood et al. 2004). In confirmation of the protective effect of caloric restriction, it has recently been shown that obesity instead is a risk factor for susceptibility to neurotoxic

insult toward DAergic neurons in obese leptin-deficient (*ob/ob*) mice (Sriram et al. 2002).

The mechanisms responsible for the protective effect of low caloric intake are not completely understood yet. Nevertheless, it is well established that exposure of neurons to a mild metabolic stress, such as dietary restriction and intermittent fasting, can protect them against excitotoxicity and other Ca^{2+} -mediated neurodegenerative processes (Calabrese et al. 2008). This type of metabolic hormesis seems mediated by transcription factors (Mattson 2008). Indeed, caloric restriction boosting the levels of some growth factors, such as brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) (Levenson and Rich 2007) glial-cell-line-derived neurotrophic factor (GDNF), and possibly BDNF (Maswood et al. 2004) enhances neurogenesis. On the other hand, obesity-associated neurodegeneration implicates an increased oxidative stress as a potential contributor (Sriram et al. 2002).

In line with these experimental results, compelling epidemiological evidence also exists. Different studies have in fact reported an elevated risk of PD in individuals with high-calorie diets (Logroscino et al. 1996; Johnson et al. 1999). Coherently, the potential association between obesity and the risk of PD has been recently shown. Hu and coworkers (2006) found in a Finnish population that excess weight, defined as a Body Mass Index (BMI) ≥ 23 , is associated with an elevated risk of PD among middle-aged men and women. The evidence that being overweight may increase the risk of PD has been confirmed in a Japanese population study (Ikeda et al. 2007) and in Japanese-American population (Abbott et al. 2003) but not in an Italian one (Ragonese et al. 2008). Population characteristics and methodological issues may partially account for the differences observed in these studies.

Notwithstanding, it is possible that higher caloric intake and consequential obesity is not actually a risk but rather an effect of PD. PD patients simply might require extra energy to cover the metabolic cost of some symptoms such as hypertonía and tremor or present a potential defect in the production of energy (Hellenbrand et al. 1996a; Logroscino and Mayeux 1997). The overall evidence from these studies appears to support a healthy imbalance of caloric intake and output, preferring a reduction of food intake.

Macronutrients

Carbohydrates

Carbohydrates are the major source of the total energy intake and they should not represent more than 50–55% of the daily caloric intake in a healthy diet. Indeed, higher carbohydrate intake has been shown to be significantly associated with diabetes mellitus and play important roles in the development

of cardiovascular disease. The importance of carbohydrate intake in PD prevention, however, is not so strong. However, data collected by the Honolulu-Asia Aging Study with 30 years of follow up showed a positive association between carbohydrate intake and risk of developing PD in a cohort of 8,006 Japanese-American men (Abbott et al. 2003). Consistently, it has been reported that PD patients eat significantly larger quantities of complex carbohydrates (Hellenbrand et al. 1996a) as well as the simple ones such as sweet foods and snacks than controls (Hellenbrand et al. 1996b). This may, however, be the result of an illness-related change in dietary habits leading to a selective recall effect, since sweet foods may enhance the transport of L-dopa across the blood–brain barrier. In conclusion, although not all the studies have revealed a strong association between high carbohydrate intake and higher risk of PD, efforts to keep carbohydrate intake from foods to approximately 55% are heartily recommended. The rationale for this is based on the well-known positive correlation between higher carbohydrate intake and diabetes type II and the new evidence of a linkage between diabetes and PD (Sandyk 1993; Hu et al. 2007a). Thus, up to 80% of PD patients are claimed to have impaired glucose tolerance (Ristow 2004), and in a cohort study in Finland, the risk of PD was 85% higher in men and women with type 2 diabetes (Hu et al. 2007b). Recently, Driver and coworkers (2008) confirmed the positive association between diabetes and PD in a large prospective study with a cohort of USA male physicians. The diagnosis of diabetes was clustered around the diagnosis of PD, suggesting that diabetes is not a preceding risk factor for PD. It is likely that the positive association may be explained by ascertainment bias or a common underlying biological mechanism. In line with the latter hypothesis, recently, it has been shown that PTEN-induced putative kinase 1 (*PINK1*) locus might be the first potential molecular link between type 2 diabetes and PD (Scheele et al. 2007). Thus, the deregulation of the whole *PINK1* locus translated to PINK1; a putative serine-threonine kinase that has been linked to a recessive form of familial Parkinsonism (Valente et al. 2004) seems to be involved in the pathophysiology of both neurodegenerative disease and type 2 diabetes. The loss of *PINK1* is not the only cause of PD, but discovering this direct link between diabetes and the regulation of the *PINK1* gene is the first example of a molecular mechanism potentially linking the two terrible illnesses, rather than just a statistical association in population studies. The next step is to find out exactly how the loss of *PINK1* actually causes neuronal cell death and hence PD.

Fat and Fatty Acids

The United States Department of Agriculture (USDA) recommends that a maximum of 30% of total daily caloric

intake comes from fat, typically somewhere between 20 and 30%. The saturated fat intake should be limited to a maximum of 10% of total calorie intake or a maximum of 1/3 of total fat intake, while the other 2/3 should consist of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Fatty acids constitute over 10% of the dry weight of the brain with PUFAs the most represented. The literature linking fat intake and PD risk is very limited and disaccording epidemiological findings also are reported (Davies 1994; Johnson et al. 1999), but a possible adverse effect of saturated fat for men could not be excluded. Indeed, two recent questionnaire-based prospective studies reported an association between high dietary consumption of saturated fat and low intake of unsaturated fatty acids with higher risk of developing PD (Chen et al. 2003; de Lau et al. 2005a). Diets with high lipid content could theoretically increase the amount of oxygen radicals by lipid peroxidation and thus increase the risk of PD (Farooqui and Horrocks 1998). On the contrary, a significant inverse association between total fat intake and PD risk has been reported in the Rotterdam study (de Lau et al. 2005b), emphasizing the selective role of unsaturated fatty acids in lowering the risk of PD. In this prospective population-based cohort study intakes of total fat, MUFAs and PUFAs were significantly associated with a lower risk of PD. An association for the essential omega3-PUFA intake by the subtype of α -linolenic acid, which makes up 88% of all omega3-PUFA intake, was observed. Linoleic acid accounts for 99% of all omega6-PUFA intake and drove the association for total omega6-PUFA. An inverse association of total fat intake with PD risk has been shown that was very similar to the one for cis-unsaturated fatty acids (MUFA and PUFA together) (Abbott et al. 2003; de Lau et al. 2005a, 2006a). A trend for an inverse association between saturated fatty acids and cholesterol and the risk of PD has also been reported (de Lau et al. 2005a). Within the several PUFA subtypes, only arachidonic acid was significantly associated with a lower PD risk. However, isocaloric replacement of polyunsaturated fat with saturated fat was associated with a significantly increased risk of PD in the men (de Lau et al. 2006a). This epidemiological data support the evidence that unsaturated fatty acids are of paramount importance for neuronal cell and brain function and in the prevention of neurodegeneration. A preponderance of research has focused on docosahexaenoic acid (DHA), an omega-3 essential fatty acid that represents between 12 and 16% of total fatty acids in gray matter lipids (Julien et al. 2006). Recently, it has been shown that a diet rich in DHA can prevent MPTP- and 6-OHDA-induced DAergic degeneration (Bousquet et al. 2008; Cansev et al. 2008). Higher DHA intake counteracted the decrease of tyrosine hydroxylase (TH)-labeled nigral cells, Nurr1 mRNA and DA

transporter mRNA levels in the substantia nigra. Although omega3-PUFA dietary treatment had no effect on striatal dopaminergic terminals, the high omega3-PUFA PUFA diet protected against the MPTP-induced decrease in DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum (Bousquet et al. 2008). Chronic diet rich in DHA does not protect nigrostriatal neurons from the initial toxic actions of 6-OHDA, but subsequently, it partially restores striatal DA, DOPAC and homovanillic acid (HVA) levels, TH levels and activity, possibly by increasing the number and/or size of nigrostriatal terminals and synapses (Cansev et al. 2008). The association between n-3 PUFA intake and PD shown by this experimental evidence needs to be confirmed by epidemiological studies.

As far as cholesterol is concerned, it is recommended to consume less than 300 milligrams per day. In a recent article, Huang et al. (2007) reported that lower serum concentrations of low-density lipoprotein (LDL)-cholesterol were associated with a higher prevalence of PD, whereas the use of cholesterol-lowering drugs was significantly associated with a decreased occurrence of PD (Huang et al. 2007). This association was found in both men and women and persisted after further adjusting for LDL-cholesterol concentration. Although these observations were made in a relatively small retrospective case-control study, they have been confirmed by a recent publication from the Rotterdam Study group (de Lau et al. 2006a). This study revealed a significant association between higher levels of total serum cholesterol and a decreased risk of PD, with analyses in quintiles showing a clear linear relation. Surprisingly, the cholesterol protection was restricted to women and the association between statin use and incidence of PD was not significant, although the trend was positive. Huang and colleagues (2007) suggested either an etiologic role for LDL-cholesterol in PD pathogenesis or a neuroprotective effect of statins. Although interesting, these results should be taken with caution; lipid-lowering drugs might be useful in the prevention of PD particularly in secondary prevention once subjects at risk of developing PD have been determined. Indeed, after the disease onset, their use may not be of further benefit, as Lieberman and colleagues (2005) have reported.

Proteins

It is recommended that a balanced diet includes the consumption of 0.8–1 g of protein per kilogram of body weight corresponding to 15–20% of total calorie intake. It is obvious that the quality of dietary proteins influences the nature and the quantities of cerebral proteins and neurotransmitters. Nevertheless, epidemiological data on the role of dietary

protein consumption in PD have been lacking. Coughlin and colleagues (1992) comparing international PD mortality data with per capita consumption of dietary protein observed a strikingly positive correlation between age-adjusted PD mortality rates and per capita consumption of total dietary protein and meat consumption. Thus, individuals consuming a high protein diet, especially meat, may be at an increased risk in either the development of PD or its severity. Nevertheless, this positive association has not been revealed in successive epidemiological studies (Anderson et al. 1999; Chen et al. 2002). In any case, the effect of proteins and essential amino acids on brain functioning, including neuronal death, is a field of fundamental and clinical research that certainly requires further research.

Micronutrients

Minerals

Iron

Dietary iron is the most important source of iron stores. The recommended dietary allowance of iron is about 8 mg per day. Several different lines of evidence suggest a primary role for iron in the pathogenesis of PD. Indeed, both neuropathological and imaging analysis of iron in the brain of PD patients has shown a selective and increased level of iron in the SNc (Lai et al. 2002). The increase of iron in the SNc DAergic neurons, possibly due to excessive dietary intake and impaired iron metabolism, could lead to a progressive degeneration of these neurons. Nevertheless, evidence that iron intake affects the risk of PD remains inconclusive. Some retrospective case-control studies found a positive association between iron intake and PD (Johnson et al. 1999; Powers et al. 2003; Gorell 1999; Logroscino et al. 1998); other studies failed instead to show any connection (Logroscino et al. 1996; Anderson et al. 1999).

Recently, a prospective study found that, although total iron intake (dietary iron and supplements) was not associated with increased PD risk, dietary iron intake alone was associated with a 30% increased risk of the disease. This association was stronger among individuals with low vitamin C intake. This was unexpectedly due to a higher risk of PD among participants who consumed large amounts of nonheme iron, the primary source of which was fortified grains/cereals, vegetables and legumes. No association was found with the intake of the better-absorbed heme iron, but the use of iron supplements was associated with a borderline increase in risk among men (Logroscino et al. 2008).

Manganese

Manganese is a mineral element that is both nutritionally essential and potentially toxic. Manganese plays an important role in a number of physiological processes as a constituent of some enzymes and an activator of others. The standard recommended daily intake of manganese is about 280–350 mg per day. Rich sources of manganese include whole grains, nuts, leafy vegetables, and teas. Manganese toxicity may arise from ore mining ferroalloy plants, the battery industry, various insecticides and welding rods. Manganese toxicity may result in multiple neurological problems such as Parkinsonism (Calne et al. 1994; Perl and Olanow 2007). It is worth noting that the pathophysiology of manganese-induced Parkinsonism is different from PD (Cersosimo and Koller 2006; Perl and Olanow 2007). Indeed, in contrast to idiopathic PD, which preferentially damages DA neurons in the SNc, manganese preferentially accumulates within and damages the pallidum and striatum, while sparing the nigrostriatal system (Bernheimer et al. 1973; Yamada et al. 1986).

In line with this evidence, it has been shown that exposure to manganese increases the risk of PD (Gorell 1999; Racette et al. 2005; Finkelstein and Jerrett 2007). In addition, one study showed that high manganese intake from nutrients and from supplements increases PD risk (Powers et al. 2003). Strikingly, when manganese is introduced with diet in combination with iron, the risk of developing PD nearly doubles compared with that of lower intake of each metal ion. Certain foods are a good source of both iron and manganese such as spinach, lima beans, peas, wheat bread, peanuts and other nuts and seeds. Multivitamins also contain iron and manganese; thus, particular attention has to be paid to these sources (food and supplements) and to avoiding an excess of the daily recommendation.

On the other hand, other epidemiological studies have failed to show any significant linkage between occupational and environmental exposure to manganese and PD risk (Semchuk et al. 1993; Hertzman et al. 1990; Tanner and Langston 1990).

Selenium

Selenium is very important in the cellular control of oxyradicals. In the mid-1980s it was proposed that selenium might be effective in the early treatment of PD (Cadet 1986). However, no significant difference has been found in the levels of selenium in the cerebrospinal fluid (CSF) of PD patients (Aguilar et al. 1998). On the other hand, decreased serum selenium levels in patients with PD have been reported (Jimenez-Jimenez et al. 1995) and dietary selenium attenuated methamphetamine-induced neurotoxicity in the

nigrostriatal DAergic system (Kim et al. 1999). Further investigation is therefore needed to ascertain the role, if any, that selenium plays in PD.

Vitamins and Antioxidants

Vitamin C (ascorbic acid), vitamin E (α -tocopherol), B2, B6, B9 (folic acid) and B12 and carotenoids such as β carotene are thought to protect cells from oxidative injury (Sies et al. 1992).

Experimental evidence exists that diets rich in tomatoes (Suganuma et al. 2002) and lycopene (Di Matteo et al. 2009) are capable of exerting neuroprotective effects in the MPTP and 6-OHDA-model of PD in rodents. Surprisingly, diets enriched with transgenic high carotene tomato increased striatal DA and DOPAC levels further supporting the importance of food in modulating the nigrostriatal DA system (Di Matteo et al. 2009).

Other animal studies showed that pretreatment with vitamin E protected both the behavioral and biochemical abnormalities produced by intrastratial 6-OHDA injections (Cadet et al. 1989) attenuating the effects of 6-OHDA on glutathione (GSH) and superoxide dismutase (SOD) (Perumal et al. 1992) but not MPTP-induced damage of dopaminergic neurons in the mouse (Itoh et al. 2006). Corroborating evidence comes from a recent meta-analysis (Etminan et al. 2005) that showed a moderate intake of vitamin E seemed to decrease the risk of developing PD by 20%, suggesting that foods rich in vitamin E may be protective. This protective influence was seen with both moderate intake and high intake of vitamin E, although the possible benefit associated with a high intake of vitamin E was not significant. The risk of PD, however, is significantly reduced among men and women with a high intake of dietary vitamin E (from foods only) (Zhang et al. 2002). Nevertheless, the DATATOP-study (Deprenyl and Alpha-Tocopherol Antioxidative Therapy of Parkinsonism) showed that Vitamin E did not delay the onset of disability associated with early, otherwise untreated PD (Parkinson Study Group 1993). In addition, no clear relation of vitamin E to the clinical progression of PD was revealed in the large prospective Honolulu–Asia Aging Study (Abbott et al. 2003). This evidence suggests that dietary intake is better than vitamin E supplementation and may show some benefit in the prevention of PD but not in PD treatment. Foods high in vitamin E include polyunsaturated plant oils, vegetables, whole-grain products, nuts and seeds. Given that these data are observational, confirmation from well-designed randomized controlled trials is necessary before suggesting changes in routine clinical practice.

As far as the B vitamins are concerned, the Rotterdam Study group showed that dietary vitamin B6 was associated with a lower risk of PD with evidence for a dose–effect

relationship (de Lau et al. 2006b). In addition, stratified analyses revealed that this association was restricted to smokers, probably accounted for by mutually reinforcing beneficial effects of smoking and vitamin B6 intake on the risk of PD. Conversely, no significant association with PD risk was observed for folate and vitamin B12, although their potential effect on PD risk is not completely ruled out (de Lau et al. 2006b). Several lines of evidence suggest neuroprotective properties of vitamin B6 through antioxidant capacities, in addition to decreasing plasma homocysteine (Mahfouz and Kummerow 2004). As oxidative stress may be prominent in PD pathogenesis, higher vitamin B6 intake may thus reduce PD risk through antioxidant effects. These findings need confirmation in observational studies or clinical trials that evaluate the relationship between levels of vitamin B6 and the risk of PD.

Supply of Antioxidants

Although antioxidants and supplements theoretically could help in the treatment of PD, the clinical data supporting their role are marginal. It is possible that the dietary intake of foods high in antioxidants may reduce the risk of developing PD. Although the available data are still limited, epidemiological studies indicate that dietary antioxidants influence the incidence of neurodegenerative disorders such as dementia (including AD) and PD (Hellenbrand et al. 1996b; de Rijk et al. 1997; Orgogozo et al. 1997; Lemeshow et al. 1998; Deschamps et al. 2001).

Coenzyme Q10

Coenzyme Q10 (CoQ10) is a vitamin-like substance used in the treatment of a variety of disorders primarily related to suboptimal cellular energy metabolism and oxidative injury. CoQ10 is a component in the electron transport system and operates between on the one hand, two flavin proteins, succinyl dehydrogenase and NADH dehydrogenase, and on the other, cytochromes, and plays an important role in ATP production during cell respiration. Immunostaining examination of the autopsied brains of sporadic PD patients has shown decreases in mitochondrial electron transfer complex I and α -ketoglutarate dehydrogenase (KGDH) activity (Hattori et al. 1991; Mizuno et al. 1994). Mitochondrial respiratory dysfunction can cause oxidative stress and this in turn may cause further mitochondrial respiratory dysfunction, consequently leading to a vicious cycle, resulting in neuronal damage. Beneficial effects of oral CoQ10 administration have been found in animal models for PD (Beal et al. 1998; Matthews et al. 1998) and in a multicenter, placebo-controlled, randomized phase II trial (Shults et al. 2002).

Surprisingly, a recent randomized, double-blind, placebo-controlled trial failed to show improvement of PD symptoms by nanoparticulate CoQ10 and did not meet its primary or secondary end points (Storch et al. 2007). The levels of CoQ10 in PD analyzed in blood samples seem to be lower than that with other neurological diseases (Matsubara et al. 1991), although this was not confirmed in another study (Jimenez-Jimenez et al. 2000). Recently, the concentrations of oxidized CoQ10 and reduced CoQ10 in the CSF of patients with PD have been examined to determine whether the balance in oxidized and reduced CoQ10 is related to the pathogenesis of PD (Isobe et al. 2007). The percentage of oxidized/total CoQ10 (%CoQ10) in the CSF was significantly higher in the untreated PD group compared with the normal control group. The % CoQ10 in the CSF of PD patients showed a significant negative correlation with the duration of illness. These findings in living patients provide in vivo evidence for a possible role for %CoQ10 in the pathogenesis in the early stages of PD development (Isobe et al. 2007).

Polyphenols

Polyphenols, divided into tannins, lignins and flavonoids, are the most abundant antioxidants in the diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate and beverages, such as tea, coffee, or wine. In recent years there has been an increasing interest in investigating their many positive pharmacological properties in preventing diseases (Scalbert et al. 2005). Several thousand molecules having a polyphenolic structure (i.e., several hydroxyl groups on aromatic rings) have been identified in higher plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens (Manach et al. 2004). More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colours of flowers, fruits and leaves. The hypothetical protective effect of dietary polyphenols against PD are supported by data obtained in laboratory animals, showing that dietary supplementation containing fruits and vegetables rich in these antioxidants is capable of attenuating the degeneration of DA neurons and symptoms caused by the neurotoxins MPTP and 6-OHDA (Datla et al. 2001, 2007; Rajeswari and Sabesan 2008). The majority of published data are about polyphenols from tea and wine and is discussed in successive paragraphs. Hitherto, only epidemiological evidence for these former flavonoids exists.

In summary, an emerging view is that polyphenolic compounds could exert beneficial effects on cells not only through their antioxidant potential but also through the modulation of different pathways such as signaling cascades, antiapoptotic processes or the synthesis/degradation

of the amyloid β peptide. Moreover, different pathways could be activated by different polyphenols present in the same extracts with beneficial interactions or synergistic effect. The elucidation of their mechanism of action should provide insight into new targets for neuroprotective drugs. The concentrations of polyphenols from diet are high enough in vivo to display pharmacological activity in the brain. Furthermore, polyphenol supplements such as green tea polyphenols or catechins, panax ginseng and ginsenoside, ginkgo biloba and EGb 761, polygonum, triptolide from tripterygium wilfordii hook, polysaccharides from the flowers of nerium indicum, oil from ganoderma lucidum spores, huperzine and stepholidines might have potential clinical benefits in attenuating the progression of PD (Chen LW et al. 2007).

Wine and Alcohol

Much of the interest about the protective effect of wine and alcohol consumption has been spurred by the dietary anomaly referred to as the “French paradox” (Renaud and de Lorgeril 1992). It was shown in the 1990s that in France the morbidity and mortality of coronary heart diseases in absolute value and in consideration of its rate to other manner of death was significantly lower than in other developed countries, despite the high consumption of fat and saturated fatty acids. The reason for this protection in the French population might be attributable in part to their high consumption of red wine (Renaud and de Lorgeril 1992). Successive epidemiological studies have shown that moderate wine consumption can also be protective against neurological disorders such as age-related macular degeneration (Obisesan et al. 1998). For example, incidence data from the so-called PAQUID (Personnes Agees Quid) study showed that people drinking 3–4 glasses of wine per day had an 80% decreased incidence of dementia and AD 3 years later, compared with those who drank less or did not drink at all (de Rijk et al. 1997; Orgogozo et al. 1997; Lemeshow et al. 1998). Recently, investigators in the Rotterdam Study (Ruitenberg et al. 2002) reported that any form of moderate alcohol would have the same beneficial effects in preventing dementia. Consistently, it has been reported that the risk of PD is significantly lower in drinkers of 2+ alcoholic drinks/day compared with abstainers and this was shown for wine and liquor drinkers (Fall et al. 1999; Ragonese et al. 2003).

The risk reduction associated with wine is possibly related to its antioxidant properties due to phenolic compounds (flavonoids and nonflavonoids). Recently, there has been an increased focus on the polyphenol *trans*-resveratrol present in grape skins with respect to its neuroprotective effects toward DAergic neuron insults (Chao et al. 2008; Blanchet et al. 2008; Okawara et al. 2007; G  linas and

Martinoli 2002). For example, it was shown that resveratrol was capable of protecting neuronal PC12 cells from insults induced by 1-methyl-4-phenyl pyridium (MPP⁺) (Gélinas and Martinoli 2002) and its daily administration prevented MPTP-induced DAergic neuronal loss in mice (Blanchet et al. 2008). Moreover, the polyhydroxylated resveratrol (oxyresveratrol) exerted neuroprotective effects against Parkinsonian mimetic 6-OHDA neurotoxicity in neuroblastoma SH-SY5Y cells (Chao et al. 2008).

In spite of these health benefits, it is noteworthy to underline that the vast majority of wines may pose a health risk to drinkers through potentially hazardous levels of metal ions as a recent research has showed (Naughton and Petr  ci 2008). Indeed, of those tested, only wines from Italy, Argentina and Brazil were found to have acceptably low levels of metals (such as iron, lead, mercury, copper, zinc and nickel) with the highest levels of contamination in wines from Hungary and Slovakia. French wines were third on the list. Therefore, a long-term exposure such as 250 ml of wine a day could expose the drinker to potentially dangerous levels of metals linked to PD (Powers et al. 2003).

Taken together, these findings raise the possibility that moderate red wine consumption, safer if Italian, may be beneficial in the prevention of PD. Moreover, its constituents such as resveratrol given as dietary supplements may be potential nutritional candidates for protection against neurodegeneration in PD.

Nervine Stimulants

Coffee

Coffee is the world's most widely used herbal infusion. A personal tribute to my compatriot Fernando Illy, who invented the espresso machine in 1904, seems necessary. Fourteen billion espresso coffees are consumed each year in Italy. In 2003, Italians consumed approximately 5.7 Kg of coffee per capita, while the Finnish up to 11 Kg (World Resource 2003). The popularity of this infusion is certainly due to caffeine's psychotropic effects. Caffeine is also present in tea, in many soft drinks, particularly energy drinks and to a small extent in dark chocolate. Compelling research has provided evidence that caffeine present in these foods, exerts anti-Parkinsonian effects, probably mediated by blocking adenosine A_{2A} receptors (Fredholm et al. 1999).

Several animal studies demonstrated that caffeine protects against neurotoxicity and the degeneration of the DAergic nigrostriatal system in several toxin models of PD. Caffeine at a dose comparable with those of typical human exposure, counteracted 6-OHDA-toxic effects, showing therapeutic benefits for akinesia (Kelsey et al. 2009), restoring the content

of monoamines and their metabolites, attenuating the apomorphine-induced rotational behavior and the number of neurons of the SNc (Aguilar et al. 2006; Joghataie et al. 2004) and preventing apoptosis in DAergic cell line PC12 (Nair 2006).

In the MPTP neurotoxin model of PD, caffeine protected blood-brain barrier dysfunction in mouse striatum (Chen et al. 2008), prevented striatal DA loss in mice (Xu et al. 2006; Chen et al. 2001) and attenuated DAergic loss (Oztas et al. 2002).

Thus, there is experimental evidence that caffeine not only provides functional protection against DAergic neurotoxicity, but also reduces the degeneration of DAergic system in models of PD. A remarkable convergence of epidemiological evidence between these animal studies exists. Indeed, recent cross-sectional and cohort studies provide evidence for a robust association between consumption of coffee or other caffeinated beverages and a reduced risk of developing PD (Powers et al. 2008; Ascherio et al. 2001; Hernan et al. 2002; Tan et al. 2003, 2007; Ross et al. 2000). Caffeine is generally thought to be the active component, given that the total caffeine intake and the intake of caffeine from noncoffee sources were found to be inversely related to PD risk, whereas no association was seen between other components in coffee and the risk of PD (Ross et al. 2000). High coffee consumption was shown to be associated with 25% risk reduction, with the highest quartile associated with about 45% risk reduction (Powers et al. 2008; Tan et al. 2008). It is noteworthy that the inverse associations of coffee, smoking and nonsteroidal antiinflammatory drugs (NSAIDs) use with PD risk are cumulative. Indeed, considering dosage of coffee and smoking, risk was reduced by 87% in individuals who smoked the most heavily, drank the most coffee and used NSAIDs (Powers et al. 2008).

Nevertheless, an inconsistent relationship between caffeine intake and the rate of progression of PD has been recently shown (Simon et al. 2008).

Tea

A few studies of Chinese (Chan et al. 1998; Tan et al. 2003, 2008) and Western (Hu et al. 2007b; Checkoway et al. 2002) populations have shown that also tea, both black and green, might be associated with a reduction in PD risk. The consumption of the number of cup-years of green tea is inversely correlated with the risk of PD (Checkoway et al. 2002; Tan et al. 2003). Drinking 23 or more cups of black tea each month (less than one per day) decreases the risk of PD by 71%. Like caffeine (Simon et al. 2008), tea does not have a disease-modifying effect in already diagnosed PD (Kandinov et al. 2007).

The beneficial role of tea is likely to depend on flavonoids (30% of the dry weight of a leaf). The polyphenol most

represented in green tea is (-)-epigallocatechin-3-gallate (EGCG). In black tea preparation, these simple flavonoids undergo oxidation resulting in the generation of more complex varieties, called thearubigins and theaflavins. A pretreatment of mice with either green tea or EGCG prevented DAergic neuron degeneration in the nigro-striatal pathway induced by MPTP (Levites et al. 2001) and attenuated the neurotoxic action of 6-OHDA in rats in vivo (Guo et al. 2007) as well as in PC12 cells (Nie et al. 2002; Levites et al. 2002a,b) and human neuroblastoma SH-SY5Y cells (Guo et al. 2005; Levites et al. 2002a,b). EGCG exerts potent DAergic neuroprotective activity by different means such as microglial inhibition (Li et al. 2006) or the blocking of DA transporter (Li et al. 2006). It is noteworthy that the neuroprotective effect of tea is not limited to the green preparation; indeed, black tea extract showed similar properties (Levites et al. 2002a, b). Black tea extract treatment induced a significant recovery in d-amphetamine-induced circling behavior, spontaneous locomotor activity, DA-D₂ receptor binding, striatal DA and DOPAC level, nigral glutathione level, lipid peroxidation, striatal superoxide dismutase and catalase activity and antiapoptotic and proapoptotic protein level. Moreover, either before or after 6-OHDA administration, black tea protected DA neurons, as evident by significantly higher number of surviving TH-immunoreactive (TH-ir) neurons, increased TH protein level and TH mRNA expression in substantia nigra (Chaturvedi et al. 2006).

Food Groups and Dietary Patterns

The imprecise nature of estimating nutrients intake over long periods, (as in the period before the onset of PD) makes the accurate study of dietary risk factors very difficult. The challenges are compounded by the fact that any number of components of each food may be the factor of interest. Therefore, better results can be obtained when we look to a higher order of classification such as food in its entirety.

Indeed, foods can be divided into groups according to their peculiar content in nutrients (macro and micro). There are various systems to group foods by different national organizations. For example, USDA (2005) divided foods into 6 major groups giving the *MyPyramid* as the visual expression of them and their relative amounts in a healthy diet considering as well the role of physical activity (Britten et al. 2006). In particular, these six groups are: grains, vegetables, fruits, fats and oils, milk (dairy food) and meat and pulses.

Dairy Food

Dairy products are the only food group that has been consistently associated with a high risk of developing PD. Indeed,

a higher consumption of milk and other dairy products has been revealed particularly in men before PD onset in all the three well-established prospective studies available so far (Chen et al. 2002; Park et al. 2005; Chen H et al. 2007). Men in the highest quintile of intake of dairy products had a 50–80% increase in PD risk compared with men in the lowest quintile (Chen H et al. 2007), men who consumed more than 0.5 l of milk per day had a 130% higher risk of PD than men who did not drink milk (Park et al. 2005). So far, the epidemiological evidence suggests that the association between dairy products and Parkinson's disease is unlikely to be due to calcium, vitamin D, or fat (Chen H et al. 2007). The exact cause is not clear; the presence of some unmeasured components or contamination of dairy with pesticides or polychlorinated biphenyls cannot be excluded. However, the overall contribution of dairy food consumption to exposure to pesticides and other neurotoxins is probably only modest. Higher dairy food consumption has been related to lower circulating levels of uric acid that has been hypothesized to be neuroprotective by preventing oxidative damage caused by reactive nitrogen and oxygen species and higher plasma levels of uric acid have been prospectively linked to a lower risk of incident PD (Davis et al. 1996; de Lau et al. 2005b; Annanmaki et al. 2007). Because of the lack of experimental data, any potential explanation is speculative. Future epidemiologic and experimental investigations are needed to further evaluate this association and to ascertain the underlying mechanisms.

Dietary Patterns

The lack of positive interaction between food groups and PD risk for all the other groups apart from dairy products is not surprising. Thus the analyses of single food type ignore important interactions between components of a diet and, more importantly, the fact that people do not eat isolated foods but rather in varying combinations that affect their absorption. For this reason, over the past decades researchers have focused their interest on dietary pattern and not on a single food or even worse on a single nutrient in relation to the prevention of diseases. Dietary patterns, thus, may be a more powerful predictor of health outcomes than any single nutrient alone. Furthermore, a dietary pattern analysis offers an approach to better understanding the complexities of eating behaviors of different population groups and subgroups and may be used to effectively develop dietary intervention strategies for target groups.

Two major dietary patterns, the so-called Western diet in opposition to the Mediterranean, have been intensively investigated. The Western pattern diet is a dietary habit characterized by high intakes of red meat, sugary drinks and desserts, high-fat dairy products, eggs and refined grains

(Cordain et al. 2005). On the other hand, Mediterranean diet is abundant in plant foods, fresh fruit, olive oil as the principal source of fat, dairy products, fish and poultry, red meat consumed in low amounts and wine consumed in low-to-moderate amounts (Willett et al. 1995). Recent evidence reported by a prospective study (Gao et al. 2007) and a meta-analysis of cohort studies, comprising more than 1.5 million healthy subjects (Sofi et al. 2008), showed that a “prudent” Western diet and even better a strict adherence to a Mediterranean diet pattern is significantly associated with a strong reduction of the incidence of PD. Moreover, the Mediterranean diet reduced the overall mortality (9%), cardiovascular mortality (9%) and cancer incidence and mortality (6%) (Sofi et al. 2008).

In the light of this new evidence, consuming a Mediterranean-like dietary pattern is strongly encouraged to reduce the risk of not only the chronic disease but also the neurodegenerative one. The neuroprotective effect of Mediterranean diet is likely due to a high intake of vegetable and fiber, PUFA and omega3 fatty acid low instead in animal saturated fat. Moreover, a diet especially rich in fish and shellfish (because of their high content of purines) may reduce the risk of PD in men by increasing the plasma urate level according to results from a large prospective study (Gao et al. 2008); a beneficial effect that is potentiated by the contemporaneous pruricemic alcohol intake.

Hence, a vegetarian diet pattern may be notably protective and therapeutically beneficial with respect to PD (McCarty 2001). Nevertheless, this is only a hypothesis since data regarding the prevalence of PD in vegetarian or vegan groups or relative clinical findings are not available as yet. Vegan diets might slow the loss of surviving DAergic neurons, retard the progression of the syndrome and promote vascular health (McCarty 2001). It is noteworthy to underline that vegetarian diets have potential health risks, namely marginal intake of essential nutrients. However, the health benefits of a well-planned vegetarian diet far outweigh the potential risks and should be encouraged.

Concluding Remarks

PD is multifactorial in etiology with genetic contributions; hence it is difficult to cure this disease targeting a single pathway. Therefore the best strategy would be to aim at preventing PD. However, unlike the chronic diseases, PD and the other neurodegenerative disorders are not largely preventable diseases. Indeed, prevention strategies can be developed only if the risk and protective factors for PD are known and unfortunately we are far from being in this position. Based on the current knowledge of risk and protective factors, prevention strategies can only be hypothesized.

For example, we can act on modifiable risks that include diet and physical inactivity (behavioral factors) and on some biological factors such as overweight, dyslipidemia, hypertension and hyperinsulinaemia that have been negatively associated with PD (Simon et al. 2007). The role of a healthy diet appears, therefore, to be of paramount importance. This assertion is based on a bulk of evidence compiled in recent years from epidemiological and animal studies, which provides compelling arguments for a beneficial effect of dietary intervention on PD and may open a new way to halt the disease and even prevent it. The findings here reviewed suggest that some natural antioxidants such as vitamins, polyphenols, resveratrol, or the essential omega-3 fatty acid DHA can prevent DA neurons from a premature death. Clearly, the available data on their potential neuroprotective effect do not provide sufficient argument for any evidence-based recommendation. However, since these nutrients have the merit of being safe, it seems reasonable to suggest that their deficiency should be avoided, at least in people at high risk of developing PD due to very old age, genetic factors, or exposure to environmental risk. This necessitates a balanced diet that contains a high intake of fruit, vegetables, legumes, whole grains, nuts, fish and poultry and a low intake of saturated fat and a moderate intake of alcohol; all qualities that can be found in the so-called Mediterranean diet. Considering that following a strictly Mediterranean diet can indeed help to prevent (13% reduction in incidence) PD (Sofi et al. 2008), it would be of great interest to determine whether this diet might have a role also in the secondary prevention in the management of early PD. It is unlikely that such a diet would be more effective than conventional drugs. However, inasmuch as PD is a gradual progressive disorder, it might be that a Mediterranean diet associated with other protective measures such as drinking coffee and tea or taking NSAIDs and lipid-lowering drugs could retard the progression of the clinical syndrome, by slowing the rate of DA cell death or by restoring function to neurons.

The following is some dietary advice aimed at reducing PD risk. The energy balance should be slightly negative. This might be obtained with a combination of a balanced low hypocaloric diet and an increase in the level of energy expenditure with physical activity. This provides a reason for not eating past the point of satiation. Nevertheless, body weight should be maintained stable and malnutrition from a severe restriction of calories should clearly be avoided.

Everything that is potentially a risk for DA neurons such as chemicals and preservatives contained in processed food, in unfiltered water and vegetable and fruits from conventional agriculture should be avoided. On the other hand, locally grown organic fresh food must be preferred. Reduction of high sugar or a low glycemic diet is something to consider. Foods high in refined carbohydrates should be reduced and

complex carbohydrates such as potatoes and brown bread, pasta and rice increased. Their low glycemic index is preferred as it is less likely to cause a high blood sugar level. Moreover, they are a good source of fiber. Dairy products should be consumed with parsimony and only the organic ones. Daily intake of vegetables and fruits should be kept high. Proteins should preferably derive from vegetable sources, less from red meat (which always should be lean, high in protein and low in evident fat) in favor of fish rich in omega3 fatty acids. Tea (black or green) and coffee should be drunk. The benefit of a good glass of red wine or a sip of whiskey with a bit of dark chocolate can be truly enjoyed. In the absence of any pathological conditions, in a healthy diet, antioxidants should derive from fresh food rather than supplements. In this way an overintake of iron and Mn, which is potentially dangerous, should be avoided.

Moreover, life modifications should begin early and should include daily moderate exercise, reduced stress, and no tobacco smoking (the benefit of nicotine might be derived from another source, maybe gum). In conclusion, we do not know if PD will be prevented, but without doubt we can reduce the known risks. Further studies are needed to validate whether a dietary intervention should be encouraged in PD prevention. Nevertheless, following a healthy diet will provide for successful brain aging and in general will increase both the health-span and the lifespan in the general population.

Conflicts of interest statement The authors declare that has no conflict of interest.

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Chapter 28

Intake of Tomato-Enriched Diet Protects from 6-Hydroxydopamine-Induced Degeneration of Rat Nigral Dopaminergic Neurons

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Abstract There is extensive evidence that oxidative damage of dopamine (DA)-containing neurons in the *substantia nigra pars compacta* (SNc) may contribute to the pathogenesis of Parkinson's disease (PD). We evaluated the potential neuroprotective effect of diets enriched with *wild-type* Red Setter (RS) tomato or transgenic High Carotene (HC) tomato, rich in β -carotene, obtained by the activation of lycopene β -cyclase (*tlcy-b*), in an animal model of PD. Male Fischer 344 rats were fed for 14 days with standard Altromin^{MT} diet, 5% RS- or 5% HC-enriched diet. Seven days after the beginning of this diet regimen, the rats were lesioned by 6-hydroxydopamine (6-OHDA) injected into the left SNc. After further 7 days, the rats were sacrificed, and DA and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in both the left (ipsilateral) and the right (contralateral) striata were measured. Striatal DA levels were reduced by $86.5 \pm 5.0\%$ in control, $86.2 \pm 5.0\%$ in HC-, and $56.0 \pm 9.0\%$ in RS-fed group. Striatal DOPAC was decreased by $85.6 \pm 5.0\%$ in controls, $83.0 \pm 6.0\%$ in HC-, and $58.9 \pm 10.0\%$ in RS-fed group. Blood was obtained from the rats on day 14 and the plasma level of lycopene and β -carotene was measured by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) for the determination of lycopene and β -carotene levels. The plasma level of lycopene was 4.7 ± 0.2 ng/ml in 5% RS-fed rats, while it was undetectable (< 2.5 ng ml⁻¹) in control and HC-fed rats. The efficacy of RS diet to preserve striatal dopaminergic innervation can be attributed to the ability of lycopene to prevent the degeneration of DA-containing neurons in the SNc.

Keywords Diet • Dopamine • Lycopene • Parkinson's disease • Striatum • β -carotene

Abbreviations

6-OHDA	6-hydroxydopamine
ANOVA	Analysis of variance
BHT	Butylated hydroxytoluene
CaMV25S	Cauliflower mosaic virus 35S
DA	Dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
GSH	Glutathione
HC	High carotene
HPLC	High performance liquid chromatography
LC-APCI-MS	Liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
PLSD	Protected least significance difference
ROS	Reactive oxygen species
RS	Red Setter
SNc	Substantia nigra pars compacta
<i>tlcy-b</i>	Tomato lycopene β -cyclase.

Introduction

Parkinson's disease (PD) is a neurological syndrome that manifests itself in varying combinations of symptoms, such as tremor at rest, rigidity, bradykinesia, and loss of postural reflexes. The neuropathological hallmark of PD is the selective degeneration of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNc) (Jellinger 1989; Scherman et al. 1989). The pathogenesis of PD favors multifactorial, genetic, and environmental events, such as formation of free radicals, impaired mitochondrial activity, increased sensitivity to apoptosis, excitotoxicity, and inflammation (Esposito et al. 2002; von Bohlen und Halbach et al. 2004). Evidence from

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studies of postmortem brain tissue suggests that the involvement of reactive oxygen species (ROS) and oxidative stress (Simonian and Coyle 1996; Ebadi et al. 1996; Jenner et al. 1992) may arise, in part, from the metabolism of DA, characterized by the production of potentially harmful ROS (Dexter et al. 1991; Jenner et al. 1992). This may be important as surviving neurons increase DA turnover to compensate for diminished synaptic transmission.

There is substantial evidence that the brain, which consumes large amounts of oxygen, is particularly vulnerable to oxidative damage (Halliwell and Gutteridge 1985). Problems occur when the production of ROS exceeds the ability of cells to defend themselves against these substances (Simonian and Coyle 1996), since oxidative stress can cause cellular damage and ROS oxidize critical cellular components, such as membrane lipids, proteins, and DNA, thereby inducing apoptosis or necrosis (Simonian and Coyle 1996; Lang and Lozan 1998; Blum et al. 2001). Cells, including neurons, normally have a number of mechanisms to resist against damage induced by ROS (Halliwell and Gutteridge 1985). The major defences are oxidant scavengers, such as glutathione (GSH), vitamin C (ascorbic acid), vitamin E (α -tocopherol), carotenoids, polyphenols, flavonoids, and antioxidant enzymes. The progressive nature of PD and the observation that neuronal degeneration in the SNc is slow and protracted (Fearnley and Lees 1991) offer good opportunities for therapeutic intervention. Hence, the current studies are focusing on finding new therapies, and very promising results are supporting the prophylactic potential of compounds normally present in or added to the diet, which could help to prevent/delay the ongoing neurodegeneration in PD (Esposito et al. 2002; Dawson and Dawson 2002; Di Giovanni 2008).

Among foods rich in antioxidants, tomatoes recently attracted great interest, since they are particularly rich in lycopene, a carotenoid showing a protective effect against heart diseases, stroke, and cancer (Rao and Agarwal 2000; Rissanen et al. 2001). In addition, lycopene protects cultured hippocampal neurons against A β and glutamate toxicity (Mattson et al. 2002), and exerts a neuroprotective effect on ischemic brain damage (Hsiao et al. 2004). In addition, ingestion of tomato powder rich in lycopene attenuated the reduction of striatal DA levels in a mouse model of PD (Suganuma et al. 2002). Tomatoes contain different classes of compounds with antioxidant properties, such as carotenoids, ascorbic acid, polyphenols, and α -tocopherol, and are a primary source of lycopene and its derivatives. The genetically modified *High Carotene* (HC) tomato was obtained by transformation of *Red Setter* (RS) tomato with *Agrobacterium tumefaciens*, containing the tomato lycopene *beta*-cyclase (*tlcy-b*) cDNA, under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter. The transgenic tomato fruit acquires the ability to convert almost all lycopene into β -carotene, and in optimal conditions, to increase the total carotenoid content of the fruits (D'Ambrosio et al.

2004). HC tomatoes may be used as an increased source of carotenoids in developing countries, similar to the Golden Rice, enriched with vitamin A (Al-Babili and Beyer 2005).

In this study, an *in vivo* rat model of 6-hydroxydopamine (6-OHDA)-induced neurotoxicity, mimicking PD, was used to compare the putative neuroprotective effects of these two tomato fruits, similar in all respects, but with specularly different contents of two important carotenoids, lycopene and β -carotene.

Experimental Procedures

Animals

Male Fischer 344 rats from Charles River Laboratories (Calco, Varese, Italy), 6–7 week-old, were used. The rats were housed in groups of five per cage, at appropriate environmental conditions ($21 \pm 2^\circ\text{C}$ room temperature, 12-h light/dark cycle, 40–60% humidity). Water was provided *ad libitum*, whereas diets were administered in controlled quantities. Procedures involving animals and their care were conducted in conformity to the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992) and international laws and policies (*EEC Council Directive* 86/609, OJ L 358,1, Dec. 12, 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication N. 85-23, 1985; and *Guidelines for the Use of Animals in Biomedical Research*, *Thromb. Haemost.* **58**, 1078–1084, 1987).

Test and Control Substances

Genetically modified HC tomato was obtained by insertion of tomato lycopene β -cyclase (*tlcy-b*) gene in the RS cultivar, using *Agrobacterium tumefaciens* as a carrier and cauliflower mosaic virus 35S (CaMV25S) as a promoter (D'Ambrosio et al. 2004). RS tomato was used for comparison. Both the tomato strains were grown in protected greenhouses, harvested at ripeness and frozen at -20°C , until use. HC tomatoes have an intense and stable orange colour, due to the activity of *tlcy-b* gene. To prepare tomato-supplemented diets, HC and RS frozen tomatoes were chopped, homogenized with a B-400 Buchi mixer (Buchi Labortechnik AG, Switzerland), and liophylized. Approximately 500 g liophylized product was obtained from 10 kg of fresh tomatoes.

Diets

Standard diet for rodents, namely Altromin^{MT}, formulated by Rieper S.p.A. (Vandoies, Bolzano, Italy), was used as a

control. HC and RS diets were formulated by Rieper, by adding appropriate amounts of lyophilized tomatoes to Altromin^{MT} diet, at the level of 5% w/w (dry weight), corresponding to 50-fold the average daily tomato consumption for humans, that is approximately 10 g kg^{-1} body weight (Zuccato, personal communication). Diets were stored in dark rooms at 20°C until use.

Experimental Study Design

The Fischer 344 male rats were randomly allocated to three groups, fed for 14 days with standard diet (control group), 5% RS, or 5% HC enriched diets, respectively, to analyze the effects of the different diets on the striatal levels of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and to determine the potential neuroprotective effect of different diets against 6-OHDA-induced nigrostriatal neurodegeneration. After a week, the rats were anesthetized with chloral hydrate (400 mg kg^{-1} , i.p.) and placed on a stereotaxic frame (David Kopf Instruments, Tujunga, USA). The skull was exposed and a burr hole was drilled through the skull at the appropriate location; a 30-gauge cannula was implanted into the left SNc (AP = -5.2 , L = 2.0 , V = -7.5 from the dura surface and with respect to the bregma), according to the atlas of Paxinos and Watson (1986) and 6-OHDA ($1 \mu\text{g} \mu\text{l}^{-1}$ of saline containing 0.01% of ascorbic acid) was unilaterally injected for 5 min into the SNc at a constant rate of $1.0 \mu\text{l min}^{-1}$ by means of a microperfusion pump (Harvard Apparatus syringe infusion pump 22, USA). To ensure maximal reproducibility, 6-OHDA solution in 0.01% ascorbate/saline was freshly prepared, filtered through a membrane filter with a pore size of $0.45 \mu\text{m}$ (type Millex®-HV, $0.45 \mu\text{m}$ Syringe filters, Japan), loaded into an Hamilton syringe and protected from light during the perfusion time. A total volume of $5 \mu\text{l}$ of 6-OHDA solution, corresponding to $5 \mu\text{g}$ of toxin, was injected into SNc; the cannula was left in place for further 5 min and then slowly removed. The incision was saturated and the animals were allowed to recover before returning to the housing facilities. Testing was carried out one week after lesioning. For both rats of the first and the second series of experiments, blood (5 ml) was withdrawn by cardiac puncture just before sacrifice on day 14 and collected in tubes containing $10 \mu\text{l}$ heparin. Plasma samples, obtained by centrifugation at $3,000 \text{ g}$ for 15 min at 4°C , were stored at -20°C until analysis.

Tissue Preparation

To establish the amount of DA and DOPAC in the striata, the rats were sacrificed by cervical dislocation. The brain was rapidly removed and immediately placed into ice-cold

saline. The right and left striata were then dissected on an ice-cooled plastic dish and stored at -80°C , until biochemical assay. To establish the extent of DA and DOPAC depletion in the left striatum, each rat served as a control for itself, as left side (ipsilateral to the lesion) DA and DOPAC levels were always compared with the right side (contralateral) DA and DOPAC levels of the same animal.

For HPLC analysis, tissue samples were weighed, transferred into 1.0 ml antioxidant solution (0.1 N HClO_4 , 0.1% $\text{Na}_2\text{S}_2\text{O}_5$, 0.01% Na_2EDTA) containing internal standard ($10 \mu\text{l}$ dihydroxybenzylamine $3 \mu\text{M}$) and afterward homogenized for 1 min by ultrasounds (vibra cellTM VC 50, Sonics & Materials Inc. Danbury, CT, USA), and then centrifuged (4224 ALC centrifuge, Milano, Italy) for 15 min at 12000 rotations/min and 4°C . The centrifugate was filtered through a membrane filter with a pore size of $0.45 \mu\text{m}$ (type Millex®-HV, $0.45 \mu\text{m}$ Syringe filters, Japan) before HPLC assay.

HPLC Assay

Dialysate samples were analyzed by reversed-phase HPLC coupled with electrochemical detection. The mobile phase was composed of 24 mM citric acid, 16 mM Na_2HPO_4 , 0.19 mM Na_2EDTA , 1.22 mM 1-eptansulfonic acid, and 17.5% methanol, adjusted to pH 2.8 with orthophosphoric acid. This mobile phase was delivered at 1 ml/min flow rate (LC-10 ADvp pump, Shimadzu Italia, Milano) through a SupelcosilTM column (LC-C8, $4.0 \times 250 \text{ mm}$, $5 \mu\text{m}$, Supelco, Bellefonte, PA, USA). Samples were injected manually into the HPLC and the detection of DA and DOPAC was carried out with a coulometric detector (Coulchem II, ESA, Bedford, MA, USA) coupled to a dual electrode analytic cell (model 5014). The potential of the first electrode was set at 0 mV and the second at +400 mV. Under these conditions, the sensitivity for DA was $0.35 \text{ pg}/20 \mu\text{l}$ with a signal-to-noise ratio of 3:1.

Extraction and Determination of Lycopene and β -Carotene in Rat Plasma Samples

Two hundred microliters rat plasma samples were transferred to 2 ml amber microcentrifuge tubes with the addition of $50 \mu\text{l}$ deuterated (d_8) β -carotene (internal standard) working solution and $350 \mu\text{l}$ ethanol containing 0.01% butylated hydroxytoluene (BHT). After 5 min vortexing, the extraction was performed by adding 1 ml *n*-hexane–0.01% BHT, then the samples were vortexed again (15 min) and centrifuged (10 min, $20,800 \text{ g}$, 4°C). The organic phase was transferred to a new 2 ml microcentrifuge tube and concentrated to dryness under a gentle stream of nitrogen. The residue was resuspended just before analysis with $100 \mu\text{l}$ dichloromethane

and then with 100 μ l mobile phase (85:5:10 v/v/v, methanol: dichloromethane:acetonitrile, all containing 0.05% BHT). Lycopene and β -carotene plasma levels were evaluated by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. The HPLC used was a Perkin Elmer series 200 quaternary pump system (Norwalk, CT, USA). Analyses were performed at room temperature using a reversed phase Luna C18(2) analytical column (250 $\times$ 3.0 mm; 5 μ m) equipped with a SecurityGuardTM Luna C18(2) cartridge (4.0 $\times$ 2.0 mm; 5 μ m) used as guard column, both purchased from Phenomenex (Torrance, CA, USA). Samples were automatically injected (20 μ l) using a Perkin Elmer series 200 autosampler, at 4°C. Chromatographic separations were carried out, at the flow rate of 0.6 ml min⁻¹, using a ternary mobile phase consisting of methanol (A) dichloromethane (B) and acetonitrile (C) all containing 0.05% BHT according to the following gradient (A:B:C): 85:5:10 to 30:40:30 in 12 min (2 min hold time), to 0:70:30 in 2 min (3 min hold time), and then to the initial composition in 2 min (12 min equilibration). The HPLC system was connected to a Sciex API 150 MCA single-quadrupole mass spectrometer (Toronto, Canada) through a Sciex Heated Nebulizer source operated at 350°C. Acquisitions were carried out in positive Selected Ion Monitoring (SIM) mode using the ions m/z 537 for lycopene and β -carotene and m/z 545 for the internal standard. The nebulizer gas (air) and the curtain gas (nitrogen) flows were both set at 2.2 l min⁻¹. The orifice and ring voltages were set at 21 and 230 V respectively. The needle current was set at 3.5 μ A. The mass spectrometer was calibrated using a polypropylene glycol standard obtained from PE Sciex setting the resolution, as peak width at half height, at 0.7 ± 0.1 u. PE Sciex Masschrom 1.1.1 software was used for instrument control and data acquisition. Peak integration of extracted ion chromatograms and all calculations of concentrations and regression parameters were performed using PE Sciex TurboQuan 1.0 software. For quantitative analyses, calibration curves in plasma in the range 4–100 ng ml⁻¹ for both lycopene and β -carotene were prepared using a pool of control plasma obtained from rats fed with standard diet, extracted, and analyzed as described for samples of treated rats. Standard curve equations were calculated using weighted (1/y) linear regressions of internal ratios (analyte/internal standard peak areas) vs. analyte concentrations. The limit of detection was 2.5 ng ml⁻¹ for both the analytes.

Data Analysis

DA and DOPAC content in each sample was expressed as ng/g wet tissue. Lycopene concentration was expressed as ng/ml of plasma. Data are mean $\pm$ SEM values of absolute

DA and DOPAC levels obtained in each experimental group. Data were processed by one-way analysis of variance (ANOVA), followed by the Fisher's protected least significance difference post hoc test (Fisher's PLSD) or Dunnett's test, to allow multiple comparisons between groups: p -values < 0.05 indicate significant difference between groups. One-way ANOVA was performed to compare the percentage depletion of DA and DOPAC (ipsilateral lesioned vs. contralateral intact striata) in each experimental group. All statistical analyses were performed with StatViewTM version 5.0.1 (SAS Institute Inc., Cary, NC, USA).

Chemicals

6-OHDA hydrobromide and BHT used for plasma samples, as well as *all trans* lycopene (98%) and *all trans* β -carotene (95%) standard compounds were from Sigma-Aldrich (St. Louis, MO, USA). *All trans* d δ - β -carotene, used as internal standard, was obtained from A.R.C. Laboratories B.V. (Apeldoorn, the Netherlands). Chemicals used for HPLC analysis and perchloric acid, as well as solvents for plasma extraction and LC-APCI-MS analyses (HPLC grade), were purchased from Carlo Erba Reagenti (Milan, Italy).

Results

Effects of Different Diet Regimens on Plasma Levels of β -Carotene and Lycopene

Groups of male Fischer 344 rats were treated either with Altromin^{MT} standard diet or with the same diet, enriched with 5% genetically modified HC tomato, or 5% *wild-type* RS tomato, for 14 days. The plasma level of lycopene at sacrifice was 4.7 ± 0.2 ng ml⁻¹ in 5% RS-fed rats ($n = 5$), while it was undetectable (< 2.5 ng ml⁻¹) in control and HC-fed rats ($n = 5$). On the other hand, lycopene was undetectable in the plasma of HC-fed rats, whereas β -carotene was below the detection limit (< 4.0 ng ml⁻¹). Food consumption and body weight increase were comparable among groups.

Effects of Different Diet Regimens on 6-OHda-Induced Depletion of Striatal Da and Dopac Levels

DA and DOPAC levels were measured in control and HC- and RS- treated rats, 14 days after the beginning of the diet treatment and 7 days after lesioning (Fig. 1). Differences in

DA and DOPAC levels were found among standard diet and HC- and RS- fed rats, with and without lesioning. DA content in the right striatum was $2.816 \pm 308 \text{ ng g}^{-1}$ of tissue in control rats, $3.262 \pm 303 \text{ ng g}^{-1}$ in RS-treated rats, and $4.600 \pm 418 \text{ ng g}^{-1}$ in HC-treated rats ($P = 0.001$ vs. controls and $P = 0.009$ vs. RS-treated rats, by Fisher's PLSD

test, $n = 9$). HC diet enhanced DA levels by $63.4 \pm 9.1\%$ compared with standard diet and by $41.0 \pm 6.6\%$ compared with RS diet.

The stereotaxic injection of $5 \mu\text{g}$ of 6-OHDA in the left SNc produced a massive loss of nigral DA neurons in the ipsilateral (lesioned side) striatal DA and DOPAC levels, compared with the contralateral (intact) side of the striatum. DA level was $297 \pm 97 \text{ ng g}^{-1}$ of tissue in the left striatum of control rats, $1.510 \pm 451 \text{ ng g}^{-1}$ in RS-treated rats ($P = 0.011$ vs. controls rats, at Fisher's PLSD test), and $417 \pm 280 \text{ ng g}^{-1}$ in HC-treated rats (Fig. 1a). The percentage decrease of DA in the ipsilateral vs. contralateral striata was $86.5 \pm 5\%$ in rats fed with standard diet (control group), $86.2 \pm 5.0\%$ in HC-fed rats, and $56.0 \pm 9.0\%$ in RS-fed rats ($n = 9$, $P = 0.006$ compared with the control group, and $P = 0.005$ vs. HC-treated rats, by Fisher's PLSD test) (Fig. 2a). The significantly reduced DA depletion may indicate

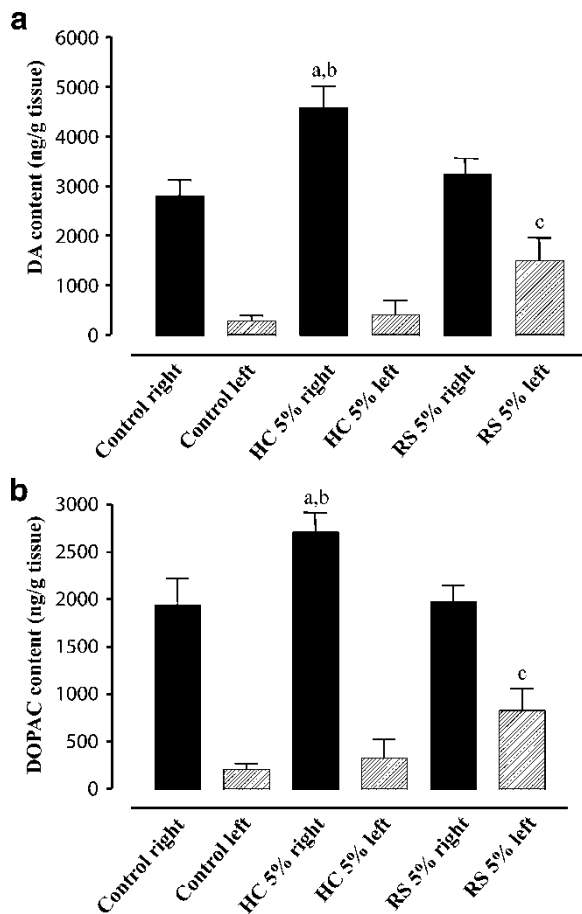


Fig. 1 (a) Effect of standard, HC or RS diet on striatal postmortem DA concentrations in rats ($n = 9$) submitted to 6-OHDA lesion in the left SNc (diagonally striped columns), and in contralateral intact side (black columns). HC diet significantly enhanced DA levels by $63.4 \pm 9.1\%$ respect to standard diet treated rats (a, $P = 0.001$) and by $41.0 \pm 6.6\%$ respect to RS diet treated rats (b, $P = 0.009$), by one-way ANOVA and Fisher's PLSD test. In the RS-fed group, the DA content of left ipsilateral striatal was significantly greater than that of the same side of the control group (c, $P = 0.011$ by Fisher's PLSD test). Data are mean $\pm$ SEM values of absolute DA levels obtained in each experimental group. (b) Effect of standard, HC or RS diet on striatal postmortem DOPAC concentrations in rats ($n = 9$) submitted to 6-OHDA lesion in the left SNc (diagonally striped columns), and in contralateral intact side (black columns). HC diet significantly enhanced DOPAC levels by $39.7 \pm 7.4\%$ respect to standard diet treated rats (a, $P = 0.0015$), and $37.2 \pm 6.3\%$ (b, $P = 0.018$) respect to RS diet treated rats. In the RS-fed group, the amount of left ipsilateral striatal DOPAC was significantly greater than that of the same(lesioned) side of the control group (c, $P = 0.029$). Data are mean $\pm$ SEM values of absolute DA levels obtained in each experimental group. One-way ANOVA and Fisher's PLSD test

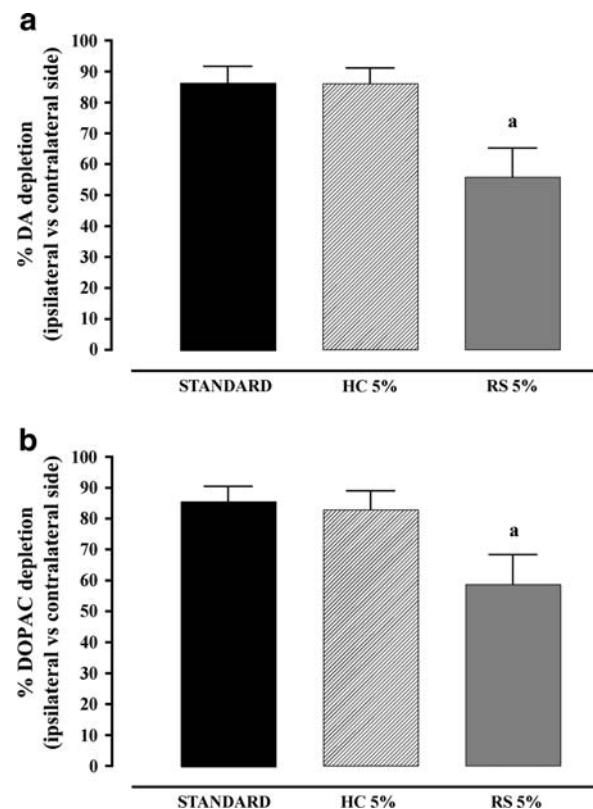


Fig. 2 (a) Effect of standard, HC or RS diet on striatal % DA depletion in rats ($n = 9$) submitted to 6-OHDA lesion in the left SNc (ipsilateral) respect to the contralateral (right) intact side. A significant difference in the decrease of DA levels in the lesioned striata was seen in RS-treated rats vs. control (a, $P = 0.006$) and vs. HC fed rats ($P = 0.005$). Data are mean ($\pm$ SEM) of DA depletion of ipsilateral vs. contralateral side. (b) Effect of standard, HC or RS diet on striatal % DOPAC depletion in rats ($n = 9$) submitted to 6-OHDA lesion in the left SNc (ipsilateral) respect to the contralateral (right) intact side. A significant difference in the decrease of DOPAC levels in the lesioned striata was seen in RS-treated rats vs. control (a, $P = 0.018$) and vs. HC fed rats ($P = 0.026$). Data are mean ($\pm$ SEM) of DA depletion of ipsilateral vs. contralateral side

a partial recovery of DA-ergic nigrostriatal function in RS-treated rats.

A similar trend was observed for DOPAC (Fig. 1b). Thus, DOPAC level was $1.945 \pm 280 \text{ ng g}^{-1}$ of tissue in the right striatum of control rats; $1.981 \pm 172 \text{ ng g}^{-1}$ in the right striatum of RS-treated rats, and $2.718 \pm 202 \text{ ng/g}$ in the right striatum of HC-treated rats ($P = 0.015$ vs. controls and $P = 0.018$ vs. RS-treated rats, by Fisher's PLSD test). HC diet enhanced DOPAC levels by $39.7 \pm 7.4\%$ with respect to control group, and by $37.2 \pm 6.3\%$ with respect to RS diet. DOPAC level in the left striatum (lesioned side) was $210 \pm 56 \text{ ng g}^{-1}$ of tissue in control rats; $834 \pm 227 \text{ ng g}^{-1}$ in RS-treated rats ($P = 0.029$ vs. controls, by Fisher's PLSD test), and $334 \pm 193 \text{ ng g}^{-1}$ in HC-treated rats (Fig. 1b). The percentage of DOPAC decrease in the ipsilateral vs. contralateral striata was $85.6 \pm 5\%$ in the control group, $83.0 \pm 6\%$ in the HC-fed group, and $58.9 \pm 10\%$ in RS-fed group ($n = 9$, $P = 0.018$ compared with the control group, $P = 0.026$ vs. HC-treated rats, by Fisher's PLSD test) (Fig. 2b).

Discussion

This study was designed to test the effect of the dietary intake of tomato in the 6-OHDA animal model of PD. The rationale of the study is based on the large amount of evidence showing that oxidative stress occurs in PD (Simonian and Coyle 1996; Ebadi et al. 1996; Jenner et al. 1992) and that nutritional antioxidants might reduce the risk or slow the progression of this neurological disease (Fahn 1991; Esposito et al. 2002). Tomato was selected as an antioxidant-rich food not only for the presence of relevant amount of bioavailable antioxidants but also because of the peculiarity of its antioxidant composition. Tomato contains, in fact, a powerful mixture of different classes of antioxidants (carotenoids, polyphenols, and vitamins) the *in vivo* potential synergic activity of which may be useful in the management of human neurodegenerative diseases (Rao and Balachandran 2002; Esposito et al. 2002; Di Giovanni 2008). Diets enriched in tomato and antioxidants have been reported to exert a neuroprotective effects in various experimental models of neurodegenerative disorders, including PD (Fall et al. 1999; Rao and Balachandran 2002; Esposito et al. 2007; Veldink et al. 2007). In particular, a four-week ingestion of an experimental diet containing 20% lyophilized tomato powder was found to prevent the neurodegenerative effect induced by the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) on nigrostriatal DA neurons (Suganuma et al. 2002). There is also evidence that lycopene affords neuroprotection against microglia activation and focal cerebral ischemia in rats (Hsiao et al. 2004). In contrast, large oral doses of β -carotene failed to prevent the

neurotoxicity of MPTP in marmosets (Perry et al. 1987), thus indicating a lack of effect of this carotenoid in the neuroprotection exerted by tomato ingestion. To clarify this point, we compared the effect of a diet enriched with *wild-type* RS tomato with that of a diet enriched with the HC tomato, a transgenic variant obtained by insertion of the *tlcy-b* gene in the RS tomato cultivar that allows an almost total conversion of lycopene to β -carotene (D'Ambrosio et al. 2004) in counteracting the toxic effect of 6-OHDA nigral infusion. Standard diet (control group) or diets enriched with 5% HC or 5% RS tomatoes were administered to male Fischer 344 rats for 14 days. A lesion was performed on day 7 and rats were sacrificed on day 14 to evaluate DA and DOPAC levels in intact and lesioned striata and the plasma level of β -carotene and lycopene.

As a first approach, the effects of tomato-enriched food on the basal levels of striatal DA and DOPAC levels were evaluated. Surprisingly, HC diet significantly enhanced striatal DA and DOPAC levels compared with both the control group and the group fed with RS tomato-enriched diet. It is possible that the increase in striatal DA levels and turnover (as indicated by the enhanced DOPAC levels) elicited by HC diet might be due to its high content in β -carotene, which is known to be cleaved by β -carotene-15,15'-dioxygenase, an enzyme expressed specifically in intestinal epithelium and in liver (Olson and Hayaishi 1965; Goodman and Huang 1965; Nagao 2004). This enzyme transforms β -carotene into retinal, which can be further metabolized to retinoic acid or retinol (Nagao 2004). The massive metabolic conversion of β -carotene to retinoids might account for the low levels of this carotenoid ($<4.0 \text{ ng ml}^{-1}$) in the plasma of rats fed with HC tomato-enriched diet. Although unexpected, the significant increase of striatal DA levels and turnover might be reconciled with several evidences indicating that retinoids play a relevant role in central nervous system development and function (Bremner and McCaffey 2008). Interestingly, several lines of evidence indicate that central dopaminergic systems are relevant targets for the action of retinoids (Bremner and McCaffey 2008). Thus, significant amounts of retinoid receptors are found in brain dopaminergic areas such as the corpus striatum and nucleus accumbens (Zetterström et al. 1999; Carta et al. 2006; Bremner and McCaffey 2008), where retinoid compounds can modulate the expression of D_2 -DA receptors (Samad et al. 1997; Krezel et al. 1998). This is consistent with the evidence that null mutations in the retinoid receptors resulted in locomotor defects related to the dysfunction of the mesolimbic DA signaling pathway and to a reduced behavioral response to cocaine (Krezel et al. 1998) and to DA receptor antagonists (Saga et al. 1999). The stimulatory effect of retinoids on D_2 -DA receptor expression (Samad et al. 1997; Krezel et al. 1998) can be reconciled with the hypothesis that retinoids are involved in the

pathogenesis of schizophrenia (Goodman 1998) and with the reported cases of affective psychosis following prolonged isotretinoin treatment (Barak et al. 2005). It is therefore tempting to speculate that prolonged exposure to retinoids might induce psychotic-like states by enhancing central dopaminergic function through several mechanisms, including the stimulation of DA synthesis, turnover, and D₂-DA expression. Consistent with this hypothesis, subcutaneous administration of 10 mg kg⁻¹ retinoic acid for four consecutive days, although it did not alter the basal DA release (as assessed by *in vivo* microdialysis), significantly potentiated stress-induced DA release in the rat prefrontal cortex (Mabrouk et al. 2003). However, 4 weeks oral isotretinoin treatment significantly increased striatal levels of the DA metabolite HVA (homovanillic acid) but it did not affect striatal DA and DOPAC levels (Ferguson et al. 2005).

The intranigral infusion of the neurotoxin 6-OHDA induced a significant decrease in DA and its metabolite DOPAC in the ipsilateral striata of controls and HC-fed rats, while in the RS group, the tomato-enriched lycopene diet partially counteracted the 6-OHDA-induced neurodegeneration, as indicated by the higher levels of DA and DOPAC found in the ipsilateral striata of RS-treated rats. The neuroprotective effect of RS diet against the 6-OHDA-induced degeneration of nigrostriatal DA neurons can be due to its content in lycopene, since this carotenoid is almost completely converted to β -carotene in HC tomato (D'Ambrosio et al. 2004). As already mentioned, our data are consistent with previous findings showing that lycopene has a neuroprotective effect against microglia activation and focal cerebral ischemia in rats (Hsiao et al. 2004). It can also protect cultured hippocampal neurons against A β and glutamate toxicity (Mattson et al. 2002). On the other hand, the evidence against a neuroprotective role of β -carotene in experimental models of PD found in this study is also strengthened by a previous study reporting that large oral doses of β -carotene failed to prevent the neurotoxicity of MPTP in marmosets (Perry et al. 1987).

Tomato and tomato products cover about 80% of dietary intake of lycopene in western countries (Nguyen and Schwartz 1998). Tomato lycopene levels vary widely, in relation to different varieties and stage of ripeness (Rao 2004). Lycopene seems to account for the health benefits of tomato-enriched diets, in the prevention of prostatic cancer, myocardial infarction, and cardiovascular diseases (Rao and Agarwal 2000; Rissanen et al. 2001). The effectiveness of lycopene is mostly due to its potent antioxidant properties (Rao 2004; Riso et al. 2004) and to the induction of phase II detoxification enzymes, converting many harmful compounds to hydrophilic metabolites that can readily be excreted from the body (Talalay 2000). Oxidative stress to dopaminergic neurons of SNc is believed to be one of the leading causes of neurodegeneration in PD. Alterations in pro- and antioxidant

molecules have been reported in postmortem tissue from individuals with PD (Dexter et al. 1994; Esposito et al. 2002).

The model of PD chosen, lesion of the SNc by 6-OHDA injection, is representative of the neuronal damage induced by free radical production (Di Matteo et al. 2006a,b) and may be particularly suitable to assess the effect of a potent free radical scavenger such as lycopene. Indeed, only the RS-enriched diet, showing consistent dosage of lycopene in plasma samples of treated rats, had a protective effect on neurodegeneration of DA-ergic neurons in the striatum. The plasma level of lycopene was comparable to that observed by Ferreira et al. (2000), upon chronic treatment of Fisher 344 rats with lycopene given in a tomato oleoresin-corn oil mixture. The lack of effect of the HC diet, rich in β -carotene, instead of lycopene, may be due to the low dosage of β -carotene or to a reduced intestinal absorption of the compound in rats (Ribaya-Mercado et al. 1989).

Recent neuroimaging and autopsy data indicated that there is a preclinical period of 4–5 years before symptoms appear and that the rate of cell loss and decline of dopaminergic function in the striatum is likely to be in the order of 10% per year, with the disease progressing relatively more rapidly during the early phases than the more advanced stages of the disease (Fearnley and Lees 1991; Brooks 1998). Both positron emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging seem to be able to detect a decline in striatal dopamine function before clinical symptoms appear (Brooks 1998), which may make it possible to begin neuroprotective intervention during the preclinical phase. Thus, at this stage, it might be particularly important to suggest a diet rich in lycopene as a preventive strategy for PD. Moreover, lycopene (or tomato enriched diet) might be considered for future clinical trials, together or as an alternative to other antioxidants such as free-radical scavengers, GSH, GSH-enhancing agents, ion chelators, and drugs that interfere with the oxidative metabolism of DA.

In conclusion, this study shows that repeated dietary intake of a transgenic tomato fruit rich in β -carotene seems to increase striatal DA and DOPAC levels and that the tomato-enriched diet exerted a beneficial effect against 6-OHDA-induced nigrostriatal lesions in rats, an experimental model of PD. The beneficial effect of tomato is most likely due to the great lycopene content of the RS-enriched diet.

Conflicts of interest statement We declare that we have no conflict of interest.

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