Cossette et al. (1999), Francois et al. (2000) and Hedreen (1999). An overview of the dopaminergic innervation in the basal ganglia is given by Smith and Kievel (2000).

6
Nigro-Subthalamic Connections in the Rat

6.1
Introduction

The STN projection neurons are glutamatergic, excitatory, and heavily innervated by widely branching axons of the substantia nigra (SN) (see Sects. 5.1 and 5.2.10, this volume). Leucine-labelled fibres of the STN follow in their projections the laminar organization of the substantia nigra's pars reticulata (Tokuno et al. 1990). However, the nigro-subthalamic connection remained controversial (see Sect. 5.2.10, this volume) due to its incomplete description in various experimental animals. Although functional dopamine receptors are expressed in the STN (see Sect. 2.3.4.1, this volume), the direct modulation of subthalamic neurons by dopamine of the substantia nigra is controversial owing to the low density of dopamine axons in the STN (see Cragg et al. 2004). Renewed tracing research was therefore carried out in the rat. To date, only an ipsilateral projection has been found for the connections between SN and STN. Using BDA, the SN-STN connection has been studied again, and a bilateral projection was established.

6.2
Materials and Methods

6.2.1
Injections

Twenty female Wistar Albino Glaxo rats weighing 200–240 g were used. The animals were anesthetized with Hypnorm (0.3 ml/kg i.p.; 0.2 mg/ml fentanyl; Ceva, Paris) and Valium (1.0 ml/kg i.p. 5 mg/ml diazepam; Hoffmann-La Roche, Basel). All rats further received a subcutaneous dose of 0.1 ml atropine sulphate (500 μg/ml) to diminish mucous secretion into the tracheo-bronchial tree. After mounting in a Narashige stereotaxic frame in the flat skull position, biotinylated dextran amine (BDA; 10%, mw 10,000; Molecular Probes Europe, Leiden, The Netherlands) dissolved in phosphate buffer (PB; 0.1 M, pH 7.2) was injected unilaterally into the SN using a vertical approach. Stereotaxic co-ordinates were obtained from Paxinos and Watson's atlas (1996). Injections were made through silicon-coated glass micropipettes (Yu and Gordon 1994), and the BDA solution was freshly prepared for each injection. Pressure injections were made using a Picospritzer, and iontophoretic injections with a Midgard CS3 iontophoretic power source (3–5 μA pulsed DC, 5 s on/off for 30 min). At the end of each injection the pipette was held in place

for 15 min to insure that the inject BDA was absorbed into the tissue, and that there was not a significant spread of the tracer within the pipette track. Survival time was 8–13 days. The rats were deeply re-anaesthetized with Nembutal (1.5 ml/kg i.p. 60 mg/ml sodium pentobarbital; Sanofi Sante, Maasluis, The Netherlands), and perfused transcardially with 100 ml of 0.9% saline, followed by 500 ml of 4% formaldehyde (Merck, Darmstadt, Germany) in water. Immediately prior to perfusion sodium nitrite (0.5 ml; 1% in water) and heparin (0.5 ml; 5,000 IE/ml; Leo Pharmaceutical Products, Weesp, The Netherlands) were injected intracardially. The brains were removed, rinsed in water for 4 h, and soaked in 10% sucrose in water overnight at room temperature. Serial sections were cut at a thickness of 40 μm on a Jung freezing microtome, and collected in PB.

6.2.2
Tracer Histochemistry

A commercial avidin-biotin-HRP complex (ABC) kit was used to visualize the BDA (Vectastain ABC Kit, Vector Laboratories, Burlingame, United States). The sections were soaked in PB containing 0.1% bovine albumin (fraction V; Sigma Chemical Co., St Louis, United States) for 30 min, and rinsed in PB for 30 min. Then the sections were incubated in the avidin-coupled biotinylated-HRP solution for 60 min on a shaker, and rinsed again in PB for 30 min. The reaction product was developed with 0.06% 3,3'-diaminobenzidine (Sigma Chemical Co., St Louis, United States) and 0.02% H_2O_2 in Tris buffer (0.05 M, pH 7.6) for 15 min. The sections were then rinsed in distilled water, mounted on chrome alum-subbed slides and dried overnight. The sections were counterstained with cresyl violet, dehydrated through graded ethanols, cleared with xylene, and cover slipped with Entellan (Merck, Darmstadt, Germany).

6.3
Results

6.3.1
Tracing Results

6.3.1.1
Appearance of Labelling

Large injections (series C5778, 5785, Fig. 25) were used to describe the overall projections of the SN. In series C5778 the injection is almost throughout the rostromedial and lateral extent of the SN, and involves the lateral SNr and SNc and the SNl, with some involvement of MRN neurons covering the dorsal surface of the SN. This injection resulted in labelling throughout STN both ipsilateral anterograde and retrograde (il) and contralateral antegrade (cl). This series will serve as the prototype for the description of the labelling observed in and around STN. The overall results

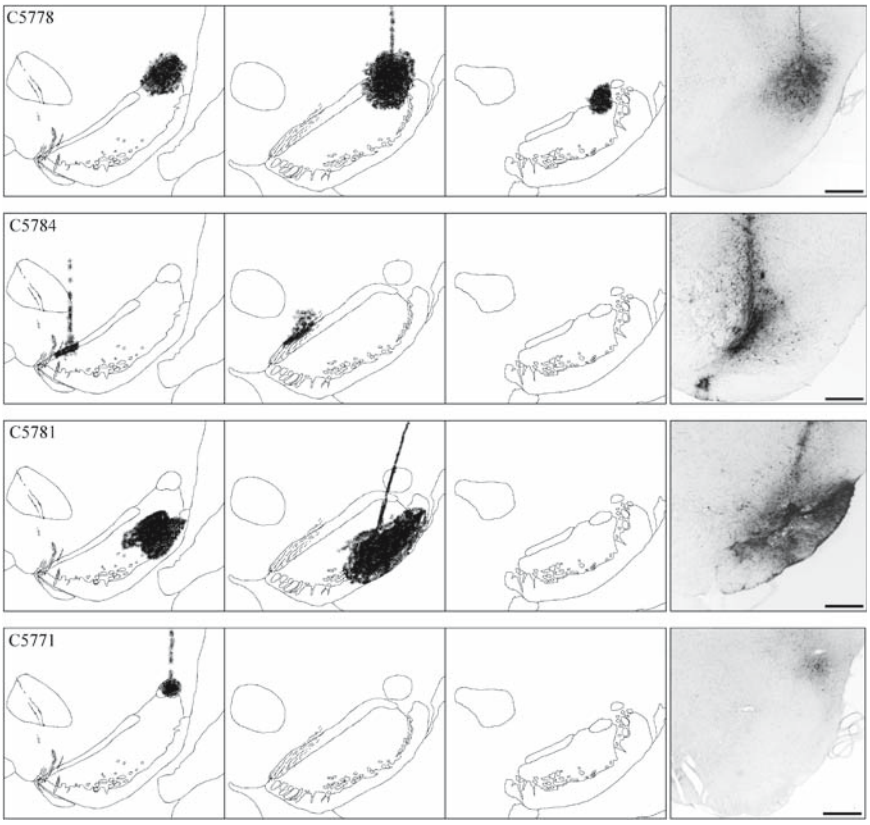

Fig. 25 Representative injection sites in the substantia nigra

from all series are shown in Table 2, while the characteristic STN connections are described separately.

Moreover, a series of injections just dorsal, rostrodorsal and rostral to the SN ($n = 8$) corroborated that the described SN connections originated in the SN.

6.3.1.2
Course and Termination of Nigrosubthalamic Connections

The largest number of nigrosubthalamic axons was observed in case 5778. The injection site of the tracer involved the mediolateral SNl, SNr and SNc (Fig. 25). The labelled axons radiate from the injection site. The axons are directed to the brain stem, and some nigrothalamic axons course dorsally towards the tegmentum, and the ascending axons to the forebrain initially take a medial course towards the prerubral area. Most of them run immediately dorsal to SN, and some axons traverse the SNc lateromedially. Few axons curve ventromedially and travel along the border between

Table 2 Connections of the substantia nigra

Afferents to substantia nigra							Efferents from substantia nigra					
SNr		SNc		SNl			SNr		SNc		SNl	
i	c	i	c	i	c		i	c	i	c	i	c
↰		↰		↰		Cortex	↲		↲		↲	
↰		↰		↰		Caudate-Putamen	↲		↲		↲	
↰		↰				Pallidum	↲		↲			
↰		↰				Accumbens			↲			
						Hippocampus			↲			
		↰		↰		Amygdala			↲		↲	
						Lateral dorsal thalamic nucleus	↲					
						Medial dorsal thalamic nucleus	↲	↲	↲			
						Ventral medial thalamic nucleus	↲	↲	↲			
						Central medial thalamic nucleus	↲		↲			
						Central lateral thalamic nucleus	↲			↲		
↰		↰		↰		Parafascicular thalamic nucleus	↲			↲		
						Paracentral thalamic nucleus	↲					
						Lateral posterior thalamic nucleus	↲					
↰		↰		↰		Lateral habenular nucleus			↲	↲		
						Dorsal lateral geniculate nucleus	↲		↲		↲	
						Zona incerta	↲					
↰		↰		↰		Subthalamic nucleus			↲			
↰	↰	↰	↰	↰	↰	Hypothalamus	↲		↲		↲	
						Superior colliculus	↲	↲			↲	
						Red nucleus	↲					
↰		↰				Entopeduncular nucleus						
						Inferior colliculus					↲	↲
						Periaqueductal gray			↲	↲		
↰		↰				Raphe dorsalis			↲			
						Cuneiform nucleus	↲					
						Mesencephalic reticular nucleus	↲		↲			
		↰	↰			Pedunculopontine tegmental nucleus	↲	↲	↲			
↰	↰	↰	↰	↰	↰	Laterodorsal tegmental nucleus	↲					
↰	↰	↰	↰	↰	↰	Parabrachial nuclei	↲		↲			
						Locus coeruleus	↲		↲		↲	
						Parvocellular pontine reticular nucleus	↲		↲			
			↰			Cerebellum		↲		↲		↲

i, ipsilateral; c, contralateral

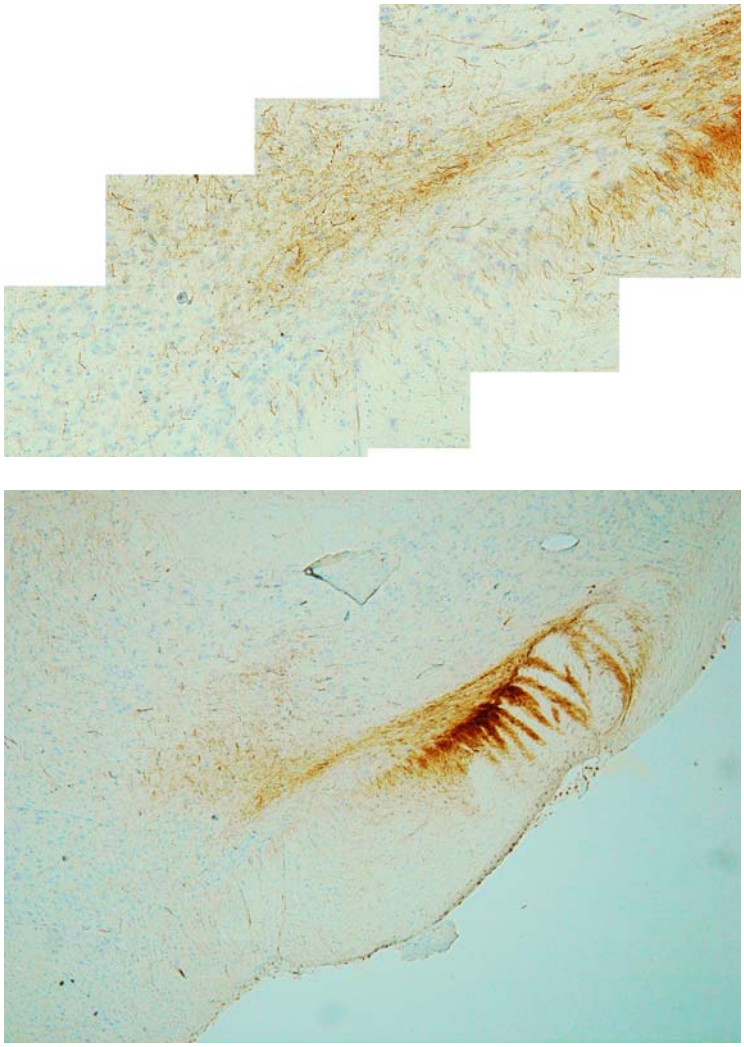

Fig. 26 Pathway of nigro-subthalamic fibres at the cerebral peduncle (series 5778)

SNr and the cerebral peduncle. Reaching the caudal pole of the STN (Fig. 26) the labelled axons enter the nucleus through its lateral wedge and from the medially running bundle, dorsal to the STN. Labelled axons also enter the STN through its ventral border, but their course is largely obscured by the numerous retrogradely labelled striatonigral axons, arranged in the bundles of the Edinger's *Kammsystem des Fusses* ("comb system of the foot"). Within the STN, especially in the lateral half of the nucleus, along with passing fibres oriented mediolaterally, there is a large amount of terminal labelling (Fig. 27). In the medial part of the STN there are mainly discrete bursts of terminal labelling. Interestingly, although the subthalamonigral projection

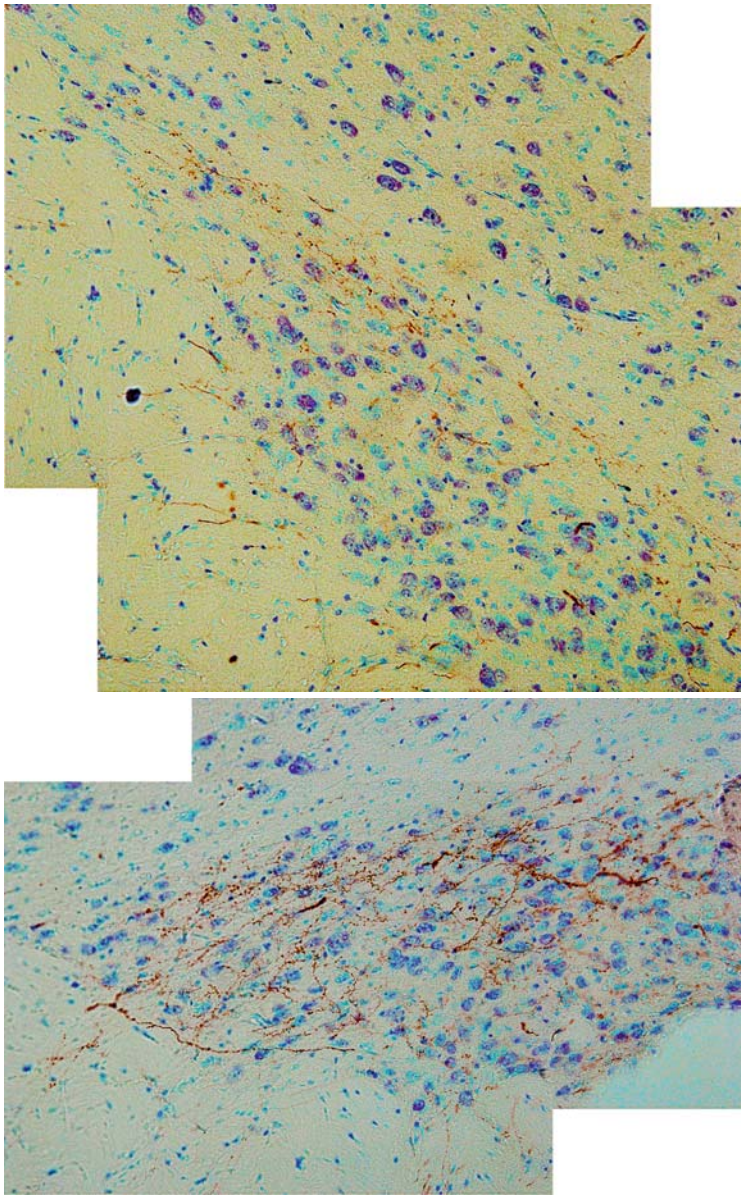

Fig. 27 Contralateral (*top*) and ipsilateral (*bottom*) nigrosubthalamic projections

is a substantial one, only few retrogradely labelled STN neurons are present, and most of them are not heavily loaded with the reaction product.

The SN axons cross the midline at several places. The most substantial component of crossed axons runs in the mesencephalic tegmentum ventral to the

periaqueductal grey. Such bundles are present through the entire rostrocaudal extent of the mesencephalon, and some fibres in the rostral mesencephalon apparently enter the STN through its dorsal border. A second component crosses the midline in the commissure of the superior colliculus and in the posterior commissure. Although some fibres bend in the ventral direction contralaterally, none of these axons appears to enter the contralateral STN. Rostral to the SN, the efferent SN axons cross the midline in the adhesio interthalamica (crossed nigrothalamic axons), and the last component of crossing axons runs in the supraoptic decussation, immediately above the optic tract. Some of these axons take a dorsomedial course towards the contralateral STN. In the contralateral STN a considerably lower number of labelled axons are seen. However, they form very distinct mediolaterally extended patches that might be followed in serial sections. Most of these discrete fields of terminal labelling are in the central and lateral portions of the STN, but also medially some terminal "whorls" are seen.

6.3.2
Injections into the SNl

A small injection (series C5771) into the SNl is without further involvement of the SNc or SNr and is placed dorsolateral of the SNc. The ilSTN contains scant fibres entering the nucleus from the dorsolateral side, and few terminations are noticed around the cells of the STN. Labelled fibres and terminations are absent in the contralateral STN. An analogous but larger injection (C5838) in the SNl, reaching the SNc, shows, however, a strong termination pattern in the ilSTN and few labelled fibres in the cl STN. These fibres entered the nucleus from its laterodorsal side. An injection (C5830) just dorsal of the SNl into the peripeduncular nucleus and inferior colliculus shows absence of labelling in both the ilSTN and the clSTN.

6.3.3
Injections into the SNr

Nearly all injections involving the SNr also touched upon the SNc, which is due to the dorsal approach of the injections. Three series (C5606, C5785, C5778) contained large injections involving not only the SNr but the SNc too. In these three series, labelling was found in the ilSTN and clSTN. From the only two selective injections into the SNr (C5835, C5781) one was mainly placed in the cerebral peduncle (C5835). Passing cortical fibres and some nigral fibres are labelled, and these fibres find their trajectory over and through the rostral top of the STN. Terminal labelling and positive fibres are found in the ilSTN. The clSTN stays free from fibres and terminal labelling in C5835. However, in C5781, with a large injection in the SNr, both ilSTN and clSTN (Fig. 28) contained labelled terminals and fibres.

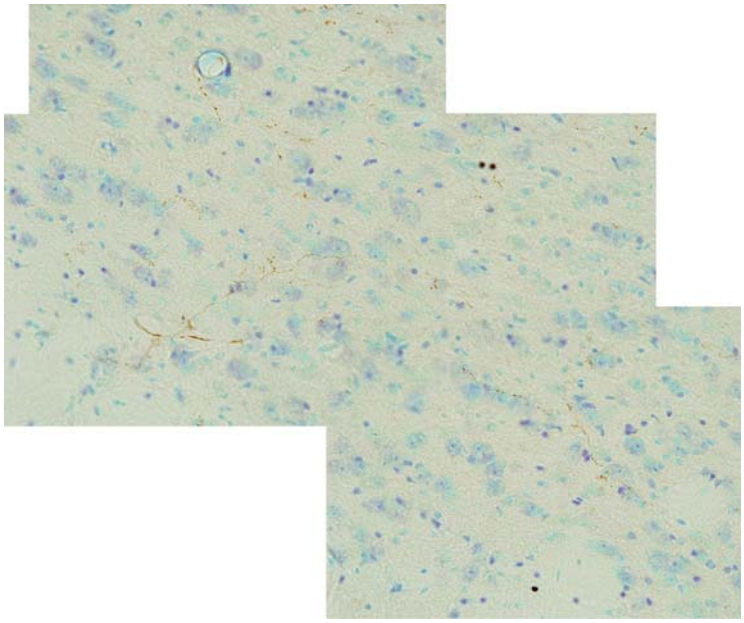

Fig. 28 Contralateral projection into the STN after SNr injection (5781)

6.3.4 Injections into the SNc

C5784 contains an injection, which is restricted to the medial part of the SNc. Heavy terminal labelling and labelled fibres are found in the ilSTN. A single retrogradely labelled ilSTN neuron was present. The clSTN contained sparse terminal labelling with a larger number of labelled fibres. The positivity was restricted to the caudal and lower middle part of the clSTN.

C5767 shows a small injection into the middle part of the SNc. Labelling (fibres and terminals) was noticed in the ilSTN. Due to the small injection size only faint terminal labelling and a few fibres are present in the middle of the clSTN. One retrograde labelled neuron is found at the transitional border of ilSN towards ilSTN. C5788 contains an injection outside the SN, but the injection touches the middle part of the SNc. The ilSTN contained heavy terminal and fibre labelling. No retrograde-filled ilSTN neurons were found. The clSTN demonstrated sparse fibre and terminal labelling in the upper caudal and middle part of the clSTN (Fig. 29).

6.3.5 Control Injections

Control injections are C5830 for the SNl injections, and C5789 and C5566 for the usual injection tract found above the SN. C5759 and C5754 cover with their injections

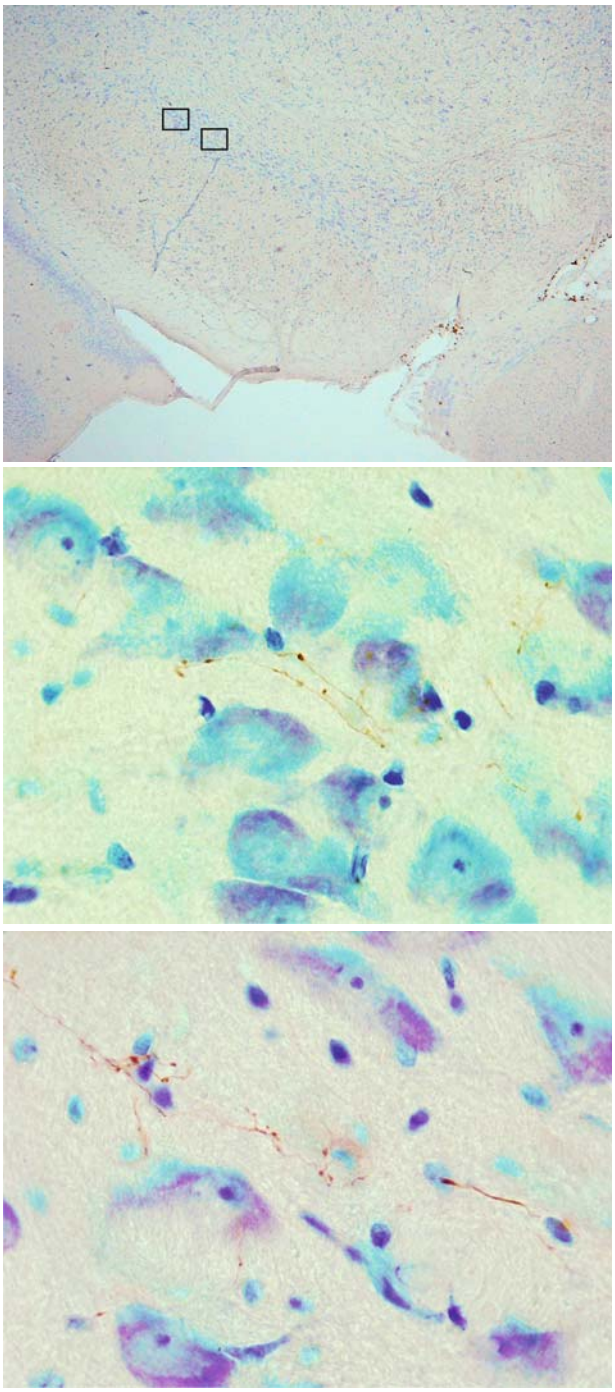

Fig. 29 C5788. The clSTN demonstrates sparse fibre and terminal labelling in the upper caudal and middle part of the clSTN

the whole SN overlaying area. The injections C5560 (caudal ruber) and C5558 (dorsal of ruber and medial of nucleus N III) involve the caudal area of the SN. No labelling was found in the clSTN.

6.4 Discussion

The present study provides data for the existence of a substantial nigrosubthalamic connection in the rat, which also emits a moderate component to the contralateral STN. Thus, two significant nuclei of the basal ganglia—the SN and the STN—are reciprocally strongly interconnected, and this STN-SN-STN loop is involved in the complicated basal ganglia circuitry, since both nuclei display a broad variety of afferent and efferent connections. Generally, the dendrites of the STN projection neurons in the rat, cat and monkey display long, thin dendrites, and in some cases the extent of the dendritic field can almost cover the overall extent of the STN (Iwahori 1978; Romansky 1982; Hammond and Yelnik 1983; Kita et al. 1983; Afsharpour 1985a; Pearson et al. 1985; Romansky and Usunoff 1985, 1987). As noticed also by Hassani et al. (1997), the extent of individual nigrosubthalamic arborizations is considerably smaller than the dimensions of the nucleus. Thus, several different nigrosubthalamic axons probably converge onto a single STN neuron, or, alternatively, a single SN axon might innervate several adjacent STN dendrites.

Many of the efferent connections of SN (to the neostriatum, thalamus, superior colliculus, periaqueductal grey, pedunculopontine tegmental nucleus, red nucleus, mesencephalic nucleus of the trigeminal nerve) are bilateral (Fass and Butcher 1981; Gerfen et al. 1982; Pritzel et al. 1983; Douglas et al. 1987; Ilinsky et al. 1987; Morgan and Huston 1990; Redgrave et al. 1992; Steiner et al. 1992; Lakke et al. 2000; and references therein). Compared to all these connections, the ipsilateral one is considerably larger, and the currently described bilateral nigrosubthalamic projection is no exception. Ipsilaterally the efferent SN axons terminate in large, profuse terminal fields, while contralaterally they terminate in discrete, sharply circumscribed patches. Although the crossed nigrosubthalamic connection is moderate, exactly by its topical distribution, its "point to point" connection is especially evident. The medial SNc projects to the contralateral medial STN, and the lateral SNc also projects mainly to the lateral half of the contralateral STN.

As reviewed in Sect. 5.2, there is already evidence for the DAergic, excitatory nigrosubthalamic connection. Its physiological significance has yet to be unravelled. Unilateral dopamine lesion has been reported to decrease the neuron discharge rate in the contralateral STN, whereas increasing this rate in the ipsilateral STN (Perier et al. 2000). Recently Carr (2002) hypothesized that this pathway might be connected with the rest tremor in Parkinson's disease, e.g. the connections of the STN with the internal pallidum, modified by SN and cortical inputs, allow for the transfer of tremorogenic activity to the thalamus.

The present data also support the suggestion of Ichinohe et al. (2000) for the existence of a moderate projection of parvalbumin containing, presumably GABAergic SN

neurons to the STN. Many of the projections of the SNr neurons—to the thalamus, tectum and reticular formation—are built by divergent collaterals of the axon of one and the same SN neuron (Bentivoglio et al. 1979; Beckstead 1983; Parent et al. 1983; Deniau and Chevalier 1992; Yasui et al. 1995; Nishimura et al. 1997). A double-labelling retrograde study might also demonstrate that the non-DAergic afferent connection to the STN is carried out by branching efferent axons of SN, as it is the case with the DAergic nigrosubthalamic tract (Prensa and Parent 2001). The STN-SNr-STN loop consists of descending excitatory component (the glutamatergic subthalamonigral tract), and ascending inhibitory component (the GABAergic nigrosubthalamic tract).

The ultrastructural morphology of the nigrosubthalamic terminal boutons and their participation in the synaptic organization of STN are still unknown. However, some of their features might be predicted. Most probably, the GABAergic nigrosubthalamic boutons share common features with other GABAergic terminals of the pallido-nigral complex: GPE, GPI and SNr, e.g. relatively large boutons containing a pleomorphic synaptic vesicle population, and contacting perikarya and large dendrites by means of symmetric synaptic specializations (Grofová and Rinvik 1974; Romansky et al. 1980a, b; Usunoff et al. 1982a; Kultas-Ilinsky et al. 1983; Williams and Faull 1988; Kultas-Ilinsky and Ilinsky 1990). Therefore, in normal STN ultrastructural material (e.g. Romansky and Usunoff 1987) the GABAergic nigrosubthalamic boutons can hardly be recognized due to the enormous number of pallido-subthalamic terminals (Romansky et al. 1980b; Usunoff et al. 1982a) and this can be reliably examined only by an electron microscopic hodological study. Since the DAergic nigrosubthalamic terminals, at least in part, represent collaterals of the nigrostriatal axons (Gauthier et al. 1999; Prensa and Parent 2001) one might expect that the nigrosubthalamic terminals are relatively small, contain pleomorphic vesicles and form symmetric synapses with various postsynaptic targets, and only rarely form asymmetric synapses with dendritic spines (Hattori et al. 1991; Groves et al. 1994; Hanley and Bolam 1997). Such tyrosine hydroxylase-positive terminals (small size, pleomorphic vesicles and symmetrical axodendritic contacts) were demonstrated in the monkey STN by Smith and Kievel (2000). Although the tyrosine hydroxylase labels noradrenergic terminals too, in all probability the vast majority of these terminals represent nigrosubthalamic terminals.

The STN is a key structure in motor control and should not be regarded only as a relay structure in the so-called indirect pathway by the parallel processing in the basal ganglia circuits (Parent and Hazrati 1995a, b). The STN can still be regarded a "control structure" lying beside the main stream of information processing (cerebral cortex > neostriatum > GPI and SNr > thalamus > cerebral cortex). However, due to its widespread efferent projections (reviewed in Sect. 5.2, this volume), the STN exerts its driving effect on most components of the basal ganglia. Its action is mediated not only by the indirect pathway (cerebral cortex > neostriatum > GPE > STN > GPI and SNr > thalamus > cerebral cortex), but also by a multitude of mono- and polysynaptic projections that ultimately reach the basal ganglia output cells (Parent and Hazrati 1995b).

DAergic medication has been shown to modulate oscillatory activity in the STN and thus may play a role in the pathology of akinesia and rigidity by affecting oscillatory synchronization in the basal ganglia (Allers et al. 2000; Brown et al. 2001; Marsden et al. 2001; Levy et al. 2002). Francois et al. (2000) reported that in the STN of Parkinson's disease patients there is a 65% loss of tyrosine hydroxylase immunoreactive axons (e.g. a 2/3 loss of the STN DAergic innervation) compared with control brains. This significant loss of DAergic innervation might directly affect the activity of the STN neurons, and might participate in the STN hyperactivity.

7 Appendix 1

7.1 Description of the Human Pathology Cases Used in this Study

Series *H3655* concerns a 66-year-old male that suffered from psychotic dementia. A high cervical transverse lesion—due to a fracture of the epistrophic dense, together with a dislocation of the atlas, long before death—was found, resulting in a total cordotomy. The patient died 6 months later of respiratory insufficiency. The cerebrum showed no distortions. The brainstem was stained with Häggqvist technique. Degeneration of the ventrolateral and the dorsal funiculi is massive (for an extensive description of this series see Marani and Schoen 2005).

Series *H5671* concerns a female patient, 56 years old, with a left-side hemiplegia due to a cerebrovascular accident; she died 6 weeks after onset of the start of the accident. After the obduction a softening was found in the right hemisphere, mainly localized in the superior and middle temporal gyri and in the lower part of the central gyri, in the inferior frontal gyrus and in the insula. It extended from there into the lentiform nucleus and corona radiata. The brain stem and spinal cord were stained for Nauta-Gygax and at regular distances frozen Häggqvist sections were made. (For an extensive description see Schoen 1969 and Voogd et al. 1998.)

H5747: The patient, male, 67 years old, died 6 weeks after an operation for otitis media from an otogenic brain abscess in the left temporal lobe. The lesion was well encapsulated in the centre of the posterior part of the temporal lobe, interrupting all afferent and efferent connections of the inferior and middle temporal gyrus. A separate small infarction just dorsal to it effectuated the same for those of the superior temporal convolution. In addition, small lesions in the brain stem interrupted the medial tegmental tract in the medial longitudinal fascicle at the level of the vestibular nuclei, and a lesion in the caudal bulbus was present in the contralateral lemniscus at the same level. The brainstem was frozen and was alternating stained according to Nauta-Gygax, Klüver-Barrera and Häggqvist techniques (see also Marani and Schoen 2005).

Series *H5889*, a Nauta-Gygax, Klüver-Barrera staining, concerns a case of colliquation necrosis in different centres in the rostral brain stem and partially in

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