Distribution and ultrastructural features of the serotonin innervation in rat and squirrel monkey subthalamic nucleus

Martin Parent, Marie-Josée Wallman and Laurent Descarries
1Department of Pathology and Cell Biology, Université de Montréal, Montreal, QC, Canada H3C 3J7
2Groupe de Recherche sur le Système Nerveux Central (GRSNC), Faculty of Medicine, Université de Montréal, Montreal, QC, Canada H3C 3J7
3Laboratoire de Neurobiologie, Centre de Recherche Université Laval Robert-Giffard, Faculty of Medicine, Université Laval, Beauport, QC, Canada G1J 2G3
4Department of Physiology, Université de Montréal, Montreal, QC, Canada

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Abstract
The main purpose of this light and electron microscopic immunocytochemical study was to characterize and compare the serotonin (5-HT) innervation of the subthalamic nucleus (STN) in rats and squirrel monkeys (Saimiri sciureus) following labeling with an antibody against the 5-HT transporter (SERT). Unbiased counts of SERT+ axon varicosities revealed an average density of 5-HT innervation higher in monkeys (1.52 x 10⁶ varicosities/mm²) than rats (1.17 x 10⁵), particularly in the anterior half of the nucleus (1.70 x 10⁶). As measured by electron microscopy, SERT+ axon varicosity profiles in the STN of both species were smaller than unlabeled profiles. The number of SERT+ profiles displaying a synaptic junction indicated that, in both rat and monkey STN, approximately half of 5-HT axon varicosities were asynaptic. In monkeys, all synaptic junctions made by SERT+ varicosities were asymmetrical, as opposed to only 77% in rats. Despite the higher density of 5-HT innervation in the anterior half of monkey STN, the ultrastructural features of its SERT+ varicosities, including synaptic incidence, did not significantly differ from those in its posterior half. These findings suggest that, throughout the rat and monkey STN, 5-HT afferents may exert their influence via both synaptic and fine subcellular localization of these 5-HT receptor subtypes. Understanding the factors governing the complex interplay between these signaling processes would greatly improve our knowledge of the physiopathology of the STN.

Introduction
The subthalamic nucleus (STN) was discovered in 1865 by Jules Bernard Luys, but its exact position in basal ganglia organization was better appreciated by Auguste Forel, who named it Luys’ body in honor of its discoverer (Parent et al., 2002). The STN is a well-delimited structure in rodents and primates, but its existence in non-mammalian vertebrates is controversial (Huber & Crosby, 1929; Jiao et al., 2000). As predicted by Luys and Forel, the STN is strategically located in the organization of the mammalian basal ganglia, with afferent projections from the globus pallidus, frontal cortex and substantia nigra, and efferent projections to all basal ganglia nuclei (for review, see Parent & Hazrati, 1995). The functional importance of the STN is evidenced by hemiballism, caused by a focal lesion of this nucleus (Jakob, 1923; Martin, 1927; Whittier & Mettler, 1949; Carpenter & Carpenter, 1951), by the drastic changes in its neuronal activity observed in animal models of Parkinson’s disease (Bergman et al., 1994; Hassani et al., 1996), and by the therapeutic effects associated with its deep brain stimulation (Benabid et al., 2009).

Like all other components of the basal ganglia, the STN receives a serotonin (5-HT) innervation (Palkovits et al., 1974) arising mainly from the dorsal raphe nucleus (Lavoie & Parent, 1990), as initially suspected from axon tracing studies (Bobbilier et al., 1976; Chan-Palay, 1977; Moore et al., 1978; Rinvik et al., 1979; Canteras et al., 1990). This 5-HT innervation has been examined in some detail, using light microscopic immunocytochemistry, in the rat, cat and monkey (Steinbusch, 1981; Mori et al., 1985; Lavoie & Parent, 1990), but such qualitative descriptions precluded rigorous interspecies comparisons.

Multiple 5-HT receptors subtypes have been recognized in the STN, including 5-HT₁₄, 5-HT₁₇, 5-HT₂₄ and 5-HT₄ (Maroteaux et al., 1992; Pompeiano et al., 1992, 1994; Compan et al., 1996; Eberle-Wang et al., 1997). Detailed information on the regional distribution and fine subcellular localization of these 5-HT receptor subtypes within the STN is lacking, but their wide variety might explain the mixed effects that 5-HT exerts on the excitability of STN neurons (Stanford et al., 2005).

As opposed to the numerous studies in the rat, cat or monkey, which have thus far reported electron microscopic data on the 5-HT innervation of the STN, such detailed information remains lacking for the squirrel monkey. We therefore undertook this study of the serotonin innervation of the STN in rats and squirrel monkeys using immunocytochemistry and electron microscopy.
innervation of most components of the basal ganglia (for review, see Descarries et al., 2010), there has not yet been a single description of the fine ultrastructural features of 5-HT axon varicosities in the STN of any species. In that context, we decided to acquire quantitative data on the regional distribution of the 5-HT innervation in STN, and to complement this information with a detailed immunoelectron microscopic analysis of the intrinsic and relational features of its axon terminals in the squirrel monkey as well as the rat. For lack of better words, ‘terminal’ and ‘varicosity’ are used as synonyms throughout this paper, without implying that these axonal enlargements containing aggregated small vesicles are endowed or not with a morphologically defined synaptic specialization (junctional complex). By comparing such data between animals in which the capacities of the motor system have considerably evolved, it was expected to gain insights into the role of 5-HT in STN function, particularly as it relates to the control of movement.

Materials and methods

Animals

The study was carried out on six adult male, Sprague–Dawley rats (Charles River, Saint-Constant, QC, Canada), weighing 300 ± 25 g, and two male squirrel monkeys, Saimiri sciureus (Charles River BRF, Houston, TX, USA), aged 8 years and weighing 800 and 850 g. Animals were housed under a 12 h light:dark cycle, with food and water ad libitum. All procedures involving animals and their care were conducted in strict accordance with the Guide to the Care and Use of Experimental Animals (Ed2) of the Canadian Council on Animal Care. The experimental protocols were approved by the Comité de Déontologie pour l’Experimenteration sur des Animaux and Université de Montréal and Université Laval. All efforts were made to minimize the number of animals used.

Tissue processing

After deep anesthesia with sodium pentobarbital (80 mg/kg, i.p.), rats were perfused transcardially with 50 mL of ice-cold sodium phosphate-buffered saline (PBS; 50 mm; pH 7.4), followed by 400 mL of 3.5% acrolein in PBS and by 600 mL of 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB; pH 7.4). Monkeys were deeply anesthetized with a mixture of ketamine (150 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.), and perfused transcardially with 500 mL of 0.9% saline (NaCl) solution at room temperature (RT) followed by 1 L of cold 4% PFA and 15% picric acid diluted in PB. Brains were rapidly dissected out, postfixed by immersion in PFA for 1 h at 4°C, and cut with a vibratome (Leica) into 60-μm-thick transverse sections, which were collected in PBS.

5-HT transporter (SERT) immunocytochemistry

Primary antibody

The polyclonal antibody against SERT (catalog # SC-1458; Santa Cruz Biotechnology, CA, USA) was raised in goat against the last 20 amino acids in the C-terminal domain of the human SERT protein. It was affinity-purified and characterized by Western blot in brain tissue. Immunoperoxidase labeling of rat and primate brain sections with this antibody allows for a light and electron microscopic visualization of axonal arborizations in the distribution and density expected from 5-HT neurons only. However, being less liable to metabolism than 5-HT, immunostaining with this antibody has been shown to be a better indicator of serotonin axons than anti-5-HT antibodies (Nielsen et al., 2006). The distribution of staining with this particular SERT antibody precisely matches that obtained with other well-characterized SERT antibodies from different sources (Pickel & Chan, 1999). Tissue processed without the primary or secondary antibody, or with the primary antiserum that had been preabsorbed with the blocking peptide (Pickel & Chan, 1999), completely abolishes the immunostaining.

In preparation for light microscopy, free-floating sections from two rats and two monkeys were sequentially incubated at RT in: (i) a blocking solution of PBS, containing 5% normal rabbit serum, 0.5% gelatin and 0.2% Triton X-100 (2 h); (ii) the same solution containing a 1 : 500 dilution of goat polyclonal antibody against SERT (overnight); and (iii) a 1 : 200 dilution of biotinylated rabbit anti-goat antibody (catalog # BA-5000; Vector Laboratories, Burlingame, CA, USA) in the same solution (1 h). After rinses in PBS, sections were incubated for 1 h at 4°C in avidin-biotin-peroxidase complex (catalog # PK-4000; Vector Laboratories) diluted 1 : 2000 in blocking solution. They were then rinsed in PBS and Tris-saline buffer (TBS, pH 7.4), and the bound peroxidase was revealed by incubating the sections for 3 min, at RT, in a 0.05% solution of 3,3′-diaminobenzidine (catalog # D5637; Sigma, St Louis, MO, USA) in 0.05 M TBS, to which 0.005% H2O2 was added. The reaction was stopped by several washes in TBS followed by PBS, and the sections mounted on gelatin-coated slides, air-dried, dehydrated in graded alcohol, cleared in toluene and coverslipped with Permount. To help in delineating the STN, adjacent sections were stained for cytochrome oxidase, according to a previously described histochemical protocol (Wong-Riley, 1979).

For electron microscopy, sections from four of the six rats and the two monkeys were prepared as above, but in the absence of Triton X-100 in all solutions. These sections were then osmicated, dehydrated in ethanol and propylene oxide, and flat-embedded in Durcupan (catalog # 44611-14; Fluka, Buchs, Switzerland) to be processed and examined as described below.

Quantitative assessment of the density of SERT immunoreactive innervation in rat and monkey STN

The number of SERT-immunostained axon varicosities was estimated in the STN of the two rats and two monkeys from which sections were prepared for light microscopic immunocytochemistry. The unbiased stereological approach schematized in Fig. 1 was used. In brief, a light microscope (Leica, DM 6000B) equipped with a digital camera (Optronics, microfire), a motorized stage (X- and Y-axes) and a Z-axis indicator (Leica Z axis control) was controlled by a computer running StereoInvestigator software (v. 7.00.3; MicroBrightField, Colchester, VT, USA). In each rat and monkey, six and eight equally spaced transverse sections across the entire right STN were taken at intervals of 120 and 180 μm, respectively. To avoid sampling bias, the first section in each series was selected at random. To achieve a more precise description of the distribution of SERT immunoreactive axon varicosities throughout the STN, the nucleus was divided in two halves along the anteroposterior axis in the rat (Fig. 1A), whereas in the monkey each of its three axes was divided in two, for a total of eight STN sectors (Fig. 1B). This was done at low magnification, by first outlining the contour of the STN on each transverse section, and then tracing a first line parallel to the lenticular fasciculus in the center of the STN, and a second line, perpendicular to it, across the center, thus delineating four sectors on each section. The anteroposterior axis was then divided in two by considering the first four transverse...
Flat-embedded SERT-immunostained sections taken through the Quadrangular pieces from the right STN were removed from four varicosities in rat and monkey STN. Ultrastructural features of SERT immunoreactive axon each animal. Used to estimate the density of axon varicosities in the entire STN of the total number calculated by the optical dissector and the volume of innervation was expressed in 10^6 varicosities per mm^3 of tissue, using ranging between 0.02 and 0.05. For each sector, the density of SERT coefficients of error (Gunderson, 1994), who found almost identical values of synaptic incidence for a large population of choline acetyltransferase-immunostained cortical varicosities examined in serial sections across their entire volume, and a randomized single section sample of these same varicosities. Statistics Two-tailed t-test and Wilcoxon signed rank test were used to detect significant differences in the density of SERT-labeled axon varicosities between anterior and posterior halves of the rat and monkey STN, respectively. Differences in density of SERT innervation of the entire STN between the rat and monkey were assessed by a two-tailed one-way ANOVA followed by Tukey's multiple comparison tests were used to detect significant differences in dimensions and synaptic incidence between SERT immunoreactive and unlabeled axon varicosities, as well as between SERT immuno-reactive varicosities from the rat and the monkey. Differences were considered statistically significant at *P* < 0.05. Statistical analysis were done using GraphPad Prism software (v. 5.01; GraphPad Software, San Diego, CA, USA). Mean and standard error of the mean are used throughout the text as central tendency and dispersion measure, respectively.
Results

Regional distribution of SERT immunoreactive innervation in the rat and monkey

As illustrated in Fig. 2A and B, the regional distribution of SERT immunoreactivity was markedly different in transverse sections across the STN of rats and monkeys. Within the rat STN, the overall density of immunostaining was relatively weak, compared with that in neighboring regions, such as the zona incerta and particularly the medial forebrain bundle, in which cross-sectioned fascicles of immunoreactive axons were visible. In the monkey STN, the immunostaining was denser, equivalent to that in the zona incerta and only slightly weaker than that in substantia nigra. A bundle of axons detached from the medial forebrain bundle could be observed to enter the STN on its dorsomedial aspect.

At higher magnification, the overall density of SERT immunostaining in each region appeared entirely imputable to that of its innervation by immunoreactive varicose axons (Fig. 2C and D). In the rat STN, these axons were thin and endowed with small varicosities. In the monkey STN, they were thicker and many of their varicosities were larger. Interestingly, in the monkey STN, both small and large varicosities could be observed along longitudinally running axon segments (Fig. 2D). Thicker and smoother axons were also seen across the nucleus. In both species, the immunoreactive varicosities were easily delineated and could thus be counted at high magnification.

Density of SERT immunoreactive innervation in rat and monkey STN

As measured with Cavalieri’s method, volumes of the right STN were 0.09 and 0.13 mm$^3$ in rats vs. 1.75 and 1.76 mm$^3$ in monkeys. Interestingly, the shape of the STN was not exactly the same in the two monkeys, one being more elongated than the other in the mediolateral axis.
The total numbers of SERT immunoreactive axon varicosities in the two rats STN were 89,364 and 143,819 vs. 2,404,705 and 2,920,787 in the monkey, corresponding to a mean density of innervation higher in the monkeys (1.52 ± 0.14) than in the rat (1.17 ± 0.07), as expressed in million of varicosities per mm³ of tissue (Fig. 3). In each animal of both species, the anterior half of the STN appeared more densely innervated than its posterior half. In the rat, the average density of SERT innervation in the anterior half of STN was 1.21 ± 0.09 vs. 1.11 ± 0.05 in its posterior half. This difference reached statistical significance in the monkey, with an average density of 1.70 ± 0.11 in the anterior half vs. 1.31 ± 0.18 in the posterior half of the nucleus (P = 0.02). No differences in density of SERT immunoreactive innervation were noticeable along the mediolateral or dorsoventral axes.

**Ultrastructural features of SERT immunoreactive axon varicosities in rat and monkey STN**

As in all other parts of rat or monkey brain where 5-HT axon terminals have been characterized at the electron microscopic level (reviewed in Descarries et al., 2010), the SERT immunoreactive axon varicosities in the STN of both species arose from thin unmyelinated axons, were generally ovoid, contained aggregated small and clear vesicles and occasional large dense-cored vesicles, and frequently displayed a mitochondrion (Figs 4 and 5). Their axoplasm was filled with a diaminobenzidine immunoprecipitate of variable density, which typically lined the plasma membrane and the outer surface of organelles. In each of the two monkeys, occasional profiles of myelinated axons displaying SERT immunoreactivity were also encountered.

As shown in Table 1, in both the rat and the monkey, the section profiles of SERT immunoreactive varicosities were significantly smaller than those of unlabeled varicosity profiles selected at random from the surrounding neuropil, regardless of the size parameter measured (P < 0.01 for short axis). Also statistically significant was the smaller area of both immunoreactive (P < 0.05) and unlabeled (P < 0.01) varicosity profiles in the rat vs. the monkey.

**SERT immunoreactive varicosity profiles displaying a synaptic junction** were observed in the STN of both species, but their frequency in single thin sections was much lower than that of unlabeled varicosity profiles (12% vs. 32% in rat and 10% vs. 42% in monkey, P = 0.01). As extrapolated to the whole volume of varicosities with the stereological formula of Beaudet & Sotelo (1981), the synaptic incidence for these SERT immunoreactive varicosities amounted to 49% in the rat and 45% in the monkey, indicating that more than half of these varicosities were in fact asynaptic (Table 2). The same extrapolation performed on the randomly selected unlabeled profiles in STN yielded considerably higher values of synaptic incidence (132% in rat and 232% in monkey), suggesting that many of these axon terminals were actually endowed with more than one junctional complex.

The SERT-immunostained varicosities appeared to make preferentially asymmetrical synaptic contact in the rat STN, whereas no symmetrical synapses were seen in the monkey STN. In both species, the synaptic targets were equally shared between dendritic branches and dendritic spines (Table 2). No synaptic contacts on cell body were observed.

In monkeys, the morphometric and junctional features of the 85 SERT immunoreactive axon varicosity profiles from the anterior half of STN were compared with the 140 profiles from its posterior half. Despite the higher density of innervation in the anterior half of STN, there were no significant differences in the ultrastructural features of SERT-immunostained axon varicosities, including synaptic incidence, between its anterior and posterior halves.

**Discussion**

This study reveals new aspects of the distribution and fine structural features of the 5-HT innervation of STN in the adult rat and squirrel monkey. By comparing quantitative data on the density of this innervation in the two species, as well as the intrinsic and relational features of its axon varicosities, it provides novel insights into the role
of 5-HT within this nucleus and, therefore, its contribution to basal ganglia functions.

**Distribution of 5-HT axon terminals in rat and monkey STN**

The experimental conditions of the present study allowed for a specific and optimal immunocytochemical detection of 5-HT axon varicosities in the STN of both the rat and the squirrel monkey. In rats, all 5-HT fibers within the STN were thin and endowed with small varicosities of relatively uniform size, suggestive of a distal unmyelinated axon arborization. In the STN of monkey, thicker and smooth axons, suggestive of fibers en passage, could also be observed across the nucleus, presumably corresponding to small myelinated axon trajectories, as previously described by Azmitia & Gannon (1983) in the medial forebrain bundle of rat and monkey. The z-depth histogram provided by StereoInvestigator indicated that the SERT-immunolabeled axon varicosities were evenly distributed throughout the thickness of the optical dissector, attesting of a complete penetration of immunoreagents. Counts of these varicosities within the different STN sectors and across the entire volume of the nucleus thus provided reliable and unbiased estimates of the density of 5-HT innervation in number of axon varicosities per volumetric unit of tissue.

In view of earlier estimates of 5-HT innervation density in various regions of the rat brain, including the cerebral cortex (Audet et al., 1989), hippocampus (Oleskevich & Descarries, 1990), substantia nigra (Moukhles et al., 1997), globus pallidus (Soghomonian et al., 1987) and striatum (Soghomonian et al., 1987; Soucy et al., 1994; Descaries et al., 1995; Mrini et al., 1995), the 5-HT innervation of the rat STN may be considered as relatively weak, whereas it appears to be moderate in the monkey, at least by comparison with other regions observed in the same sections. Correlations between such estimates for different brain areas and corresponding biochemical measurements of 5-HT concentration (Palkovits et al., 1974) have indicated that the number of 5-HT axon varicosities is a good predictor of the local concentration of 5-HT.

The lack of statistically significant differences in number of 5-HT varicosities between the anterior and posterior halves of the rat STN was in keeping with the earlier report by Mori et al. (1985) of a diffuse distribution of 5-HT immunoreactive fibers throughout the rat STN. Similarly, the greater density of 5-HT axon varicosities in both the anterior (140%) and posterior (118%) halves of the squirrel monkey compared with rat was consistent with the report by these authors of a stronger innervation in the STN of Macaca fuscata (Old World monkey) compared with rat. However, at variance with our observation of a significantly greater density of 5-HT innervation in the anterior vs. posterior half of the squirrel monkey, Mori

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**Fig. 4.** Examples of SERT immunoreactive axon varicosities from the rat STN, as visualized by electron microscopy after labeling with the immunoperoxidase-diaminobenzidine technique. In this and the following plate, all pictures are shown at the same magnification to facilitate the comparison between rat and monkey. Note the generally smaller size of these axon varicosities in the rat compared with the monkey (see Fig. 5). The SERT immunoreactive varicosity profiles in (A) and (B) are both observed in synaptic contact (between small arrows), with a dendritic spine (sp) and a dendritic branch (db), respectively. (C) Two immunoreactive axon varicosities are juxtaposed to a longitudinally sectioned unlabeled dendrite (d), but neither displays any area of synaptic membrane specialization. The SERT-immunostained axon varicosity in (D) is juxtaposed to an unlabeled myelinated axon (a). Scale bar: 1 μm.
et al. had described the 5-HT innervation of *Macaca fuscata* STN as more abundant in the ventromedial portion of the nucleus, whereas Lavoie & Parent (1990), in their earlier study in the squirrel monkey, had found it rather evenly distributed throughout the anteroposterior extent of the nucleus, but slightly denser laterally.

An earlier comparative neuroanatomical study has emphasized the remarkable increase of STN volume and neuron number from rodents to primates (Hardman et al., 2002). The STN was then shown to be 300 times larger and to contain about 25 times more neurons in humans than rats. Such a differential increase appears to have progressively led to a constant reduction of the STN neuronal density during evolution (Marani et al., 2008). The neuronal density in the rat STN reported in the above-mentioned study combined with our own estimate of the density of the 5-HT innervation in the same species indicate that there exist approximately 42 5-HT axon varicosities per STN neuron in rats. A similar extrapolation for squirrel monkeys is precluded by the lack of information on the volume and neuronal density of the STN in that species. Nevertheless, the relatively low neuronal density reported in primates (marmosets, macaques, baboons, humans; Hardman et al., 2002), coupled to the high density of 5-HT innervation noted in the squirrel monkey, allows to infer that the ratio of 5-HT varicosities per STN neuron should be higher in the squirrel monkey than rat, perhaps indicative of a stronger 5-HT drive of the STN neurons in this species. Such a ratio is, however, likely to vary from one region of the STN to another in both rodents and primates, because the neuronal density is reportedly greater at the mid anteroposterior level of the nucleus compared with its anterior and posterior levels.

Table 1. Morphometric features of SERT-immunostained vs. randomly selected unlabeled axon varicosities in the rat and monkey STN

<table>
<thead>
<tr>
<th>Axon varicosity parameter</th>
<th>Rat (n = 4)</th>
<th>Monkey (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SERT</td>
<td>Unlabeled</td>
</tr>
<tr>
<td>Number examined</td>
<td>218</td>
<td>218</td>
</tr>
<tr>
<td>Short axis (µm)</td>
<td>0.41 ± 0.03**</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>Long axis (µm)</td>
<td>0.75 ± 0.07*</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>1.87 ± 0.09</td>
<td>1.84 ± 0.08</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>0.58 ± 0.05*</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td>Area (µm²)</td>
<td>0.27 ± 0.04**</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>Those with mitochondria (%)</td>
<td>44 ± 3</td>
<td>50 ± 5</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. The unlabeled profiles were selected at random from the same micrographs displaying the SERT profiles, as explained in Materials and methods. *P < 0.05 and **P < 0.01 for SERT vs. unlabeled. 1P < 0.05 and 2P < 0.01 for rats vs. monkeys. SERT, serotonin transporter.

Fig. 5. Examples of SERT immunoreactive axon varicosities from the monkey STN. (A and B) Both SERT immunoreactive axon varicosities are seen to make a synaptic contact with a dendritic branch (db). The junctional complexes (between small arrows) are clearly asymmetrical. (A) Note the presence of a subjunctional apparatus, as occasionally observed in the two monkeys. (C and D) The immunoreactive varicosities are relatively large and do not display any area of synaptic membrane specialization: the one in (D) shows many mitochondria, and is directly apposed to other, unlabeled, axon varicosities (av). Scale bar: 1 µm.
already been described in the human STN (Morel 2003). The compartmen
talization of calcium-binding proteins distribution has been compared with rat. In this regard, it should be noted that some more specific and ordered spatial organization in the STN of monkey innervation along its anteroposterior axis, evokes the possibility of a Yelnik, 1983). Such a ratio between the size of the STN and that of the monkeys there is room for five non-overlapping neurons (Hammond & Harris, 1999). In this context, possible effects of high-frequency stimulation on 5-HT neurotransmission in the STN might affect fiber tracts in the vicinity of the STN. Moreover, some of these studies have suggested that current spread along the STN itself, as well as in the medial forebrain bundle, should not be excluded.

posterior poles (Hardman et al., 2002). A close attention should therefore be paid to such an heterogeneity in addition to the regional distribution of 5-HT receptors, if one hopes to derive the full functional significance of the quantitative estimates of 5-HT innervation density provided in the present study. Morphological analyses of the dendritic arborization of STN neurons have revealed many similarities between rats and monkeys. In both species, the dendritic fields of STN neurons are ellipsoidal in shape, measuring approximately 1000 × 600 × 300 μm, with the longest axis being parallel to the anteroposterior axis of the nucleus (Yelnik & Percheron, 1979; Hammond & Yelnik, 1983). However, a striking interspecies difference emerges when one compares the proportion of the STN occupied by these dendritic fields. In rats, the dendritic field of a single neuron may cover the whole nucleus, whereas in macaque monkeys there is room for five non-overlapping neurons (Hammond & Yelnik, 1983). Such a ratio between the size of the STN and that of the dendritic domain of its neurons, combined with the differential 5-HT innervation along its anteroposterior axis, evokes the possibility of a more specific and ordered spatial organization in the STN of monkey compared with rat. In this regard, it should be noted that some compartmentalization of calcium-binding proteins distribution has already been described in the human STN (Morel et al., 2002).

The greater density of 5-HT innervation in the anterior vs. posterior half of the monkey STN was also noteworthy in the context of deep brain stimulation as a means to alleviate the motor symptoms of Parkinson’s disease. In clinical studies, the most effective location of the electrode has been shown to be the anterodorsal part of the STN (Lanotte et al., 2002; Saint-Cyr et al., 2002; Voges et al., 2002). Moreover, some of these studies have suggested that current spread from the electrode might affect fiber tracts in the vicinity of the STN. In this context, possible effects of high-frequency stimulation on 5-HT axons running through the STN itself, as well as in the medial forebrain bundle, should not be excluded.

### Table 2. Junctional features and SERT-immunostained vs. randomly selected unlabeled axon varicosities in the rat and monkey STN

<table>
<thead>
<tr>
<th>Axon varicosity parameter</th>
<th>Rat (n = 4)</th>
<th>Monkey (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SERT</td>
<td>Unlabeled</td>
</tr>
<tr>
<td></td>
<td>SERT</td>
<td>Unlabeled</td>
</tr>
<tr>
<td>Synaptic incidence (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single sections</td>
<td>12 ± 3**</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Whole volume</td>
<td>49 ± 14*</td>
<td>132 ± 13</td>
</tr>
<tr>
<td></td>
<td>45 ± 18***</td>
<td>232 ± 25</td>
</tr>
<tr>
<td>Length of synaptic junctions (μm)</td>
<td>0.19 ± 0.02*</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Synaptic target (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dendritic branches</td>
<td>61 ± 17</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>Dendritic spines</td>
<td>39 ± 17</td>
<td>30 ± 10</td>
</tr>
<tr>
<td></td>
<td>40 ± 3</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>Junctions (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symmetrical</td>
<td>23 ± 13**</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Asymmetrical</td>
<td>77 ± 13**</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Symmetrical with</td>
<td>67 ± 33</td>
<td>71 ± 11</td>
</tr>
<tr>
<td>dendritic branches</td>
<td>0</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>Asymmetrical with</td>
<td>41 ± 16</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>dendritic spines</td>
<td>60 ± 3</td>
<td>65 ± 15</td>
</tr>
</tbody>
</table>

Data are from the same sectional profiles varicosities as in Table 1 (means ± SEM). The varicosity profiles were classified as showing or not a synaptic junction according to the criteria described in Materials and methods. The synaptic incidence for the whole volume of varicosities was extrapolated by means of the formula of Beaudet & Sotelo (1981), using the long axis as diameter of profiles (Umbrico et al., 1994). *P < 0.05, **P < 0.01 and ***P < 0.001 for SERT vs. unlabeled. SERT, serotonin transporter.

Ultrastructural characteristics of 5-HT axon varicosities in rat and monkey STN

In both species, the SERT immunoreactive varicosities were undistinguishable from unlabeled varicosities by their shape, vesicular content or frequency of mitochondria in single thin section. Yet, these varicosities were markedly smaller than their unlabeled counterparts (profile area 53% and 57% smaller in the rat and the monkey, respectively), even though both the SERT immunoreactive and unlabeled varicosities appeared to be significantly larger in the monkey than the rat.

It has been suggested that the potential ‘synaptic efficacy’ of axon terminals might be directly correlated with their size (Pierce & Lewin, 1994). This size principle is based on the idea that ultrastructural features underlying physiological strength are linearly correlated to the volume of an axon varicosity. It includes vesicle number, active zone number and area, and mitochondrial volume. According to this principle, 5-HT axon varicosities in the monkey STN should show greater ‘efficacy’ than in the rat, perhaps in keeping with the more elaborate motor programs executed by the monkey. However, it must be noted that the randomly selected unlabeled axon varicosities from the monkey STN were also larger than those from the rat. Whether this difference reflects a generally larger size of axon varicosities in primates than rodents might deserve examining. It should also be pointed out that, until now, the size principle has been demonstrated only for axon terminals containing amino acid transmitters (Lisman & Harris, 1993; Pierce & Lewin, 1994). Whether this principle also applies to axon varicosities that are only partially synaptic remains an open question. In this regard, it would be of interest to know if the concentration of 5-HT within STN varicosities is indeed related to their size. Values obtained following biochemical micromeasurements in rodents (Palkovits et al., 1974) allow us to estimate that single axon varicosities in rat STN contain about 0.96 fg of 5-HT (for the basis of this calculation, see Lapierre et al., 1973; Doucet et al., 1988), but the lack of such precise biochemical data in primates precludes a similar evaluation for the monkey STN.

The present study reveals that approximately half of the 5-HT axon varicosities in both rat and monkey STN are without synaptic membrane specialization. This relatively low synaptic incidence of the 5-HT varicosities was all the more striking as the unlabeled varicosities randomly selected from the same thin sections displayed a synapse in unusually high proportion (> 100%, when calculated by the formula of Beaudet & Sotelo, 1981), suggesting that many were endowed with more than one junctional complex.

The largely asynaptic character of many chemospecific innervations in the CNS has been viewed as morphological evidence for the existence of diffuse transmission by such neuronal systems, in addition to their synaptic mode of transmission (reviewed in Descarries & Mechawar, 2008). In the case of 5-HT, as for acetylcholine and dopamine, it has also led to the suggestion that a low, ambient level of transmitter might permanently exist in the extracellualr space, the fluctuations of which could regulate a variety of physiological processes mediated by 5-HT and other transmitter receptors widely distributed on neuronal, glial and vascular elements (Descarries et al., 1997). Convincing electrophysiological evidence for a regulatory role of ambient 5-HT has already been obtained in the rat substantia nigra (Bunin et al., 1998). Thus, in the rat and monkey STN, diffuse as well as synaptic transmission could contribute to a modulatory control of STN function by 5-HT. The different mechanisms by which 5-HT release is regulated in the diffuse and synaptic modes of transmission as well as the 5-HT receptors that could be associated with these modes of transmission remain to be determined.
In both the rat and the monkey, about 60% of the synaptic 5-HT varicosities targeted dendritic branches as opposed to dendritic spines. No axo-somatic synapses were observed. All synaptic junctions made by 5-HT varicosities in the monkey appeared to be asymmetrical, as opposed to only 77% in the rat. This latter difference had to be regarded with caution, however, as it was based on low numbers of varicosities displaying a junctional specialization, and which were examined in single thin sections only. For these reasons, it was deemed hazardous to speculate on its possible significance, or to assume that it reflected differences in precise anatomical origin between the rat and the monkey.

Functional considerations

The STN sends glutamatergic projections to several nuclei, including the pallidum, substantia nigra and striatum. In turn, it receives various chemospecific inputs from the globus pallidus, center median/para-fascicular thalamic complex, substantia nigra, midbrain raphe nuclei and pedunculopontine tegmental nucleus. In addition, a direct projection from the cerebral cortex has been documented in the rat (Kitai & Deniau, 1981; Afsharpour, 1985; Canteras et al., 1990) as well as in the monkey (Kunzle & Akert, 1977; Carpenter et al., 1981). However, the cortico-subthalamic projection has been shown to be somatotopically organized only in monkeys (Kunzle, 1978; Monakov et al., 1978; DeLong et al., 1985), a finding that is in keeping with the topographical distribution of somatodendritic domains of monkey STN neurons discussed above (Hammond & Yelnik, 1983). With all these diverse inputs, the net activity of STN neurons is likely to result from a complex interaction between various chemospecific neuronal systems. Synaptic and diffuse 5-HT transmission, and hence fluctuations in the ambient level of 5-HT within the STN, might not only influence the activity of STN neurons directly, but also act locally on these afferent systems.

In addition to a variety of postsynaptic effects on the excitability of STN neurons (Stanford et al., 2005; Xiang et al., 2005), in vitro experiments conducted in rodents have suggested that 5-HT inhibits the synaptic activation of STN neurons through axodendritic (pre-synaptic) 5-HT1B receptors (Shen & Johnson, 2008). Interestingly, it appears that, at low concentration, 5-HT reduces glutamate-mediated excitatory postsynaptic currents in STN neurons, whereas a higher concentration is needed to inhibit γ-aminobutyric acid (GABA)-mediated inhibitory postsynaptic currents (Shen & Johnson, 2008). To what extent these effects might reflect the synaptic vs. diffuse mode of transmission by 5-HT is currently unknown, but their dependence on the local concentration of 5-HT strongly suggests that the level of 5-HT maintained in the STN might actually determine the role of this neurotransmitter in the regulation of motor behavior. Indeed, pharmacological manipulations of 5-HT1A and 5-HT1B receptors in the STN have been shown to alter locomotor activity in rodents (Martinez-Price & Geyer, 2002), whereas significant attenuation of levodopa-induced dyskinesias in 6-OHDA-lesioned rats were obtained following local administration of a 5-HT1A agonist (Marin et al., 2009). These findings underline the importance of the 5-HT innervation of the STN in the control of motor behavior and may lead to novel pharmacological approaches for the treatment of motor disorders involving the STN.

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Abbreviations

5-HT, serotonin; PB, sodium phosphate buffer; PBS, sodium phosphate-buffered saline; PFA, paraformaldehyde; RT, room temperature; SERT, serotonin transporter; STN, subthalamic nucleus; TBS, Tris-saline buffer.

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