QUANTITATIVE AND MORPHOMETRIC DATA INDICATE PRECISE CELLULAR INTERACTIONS BETWEEN SEROTONIN TERMINALS AND POSTSYNAPTIC TARGETS IN RAT SUBSTANIA NIGRA


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Abstract—We have quantified the density of serotonin axonal varicosities, their synaptic incidence and their distribution among potential targets in the pars reticulata and pars compacta of the rat substantia nigra. Serotonin axonal varicosities, counted at the light microscopic level following in vitro [3H]serotonin uptake and autoradiography, amounted to 9 × 10⁶/mm² in the pars reticulata and 6 × 10⁶/mm² in the pars compacta, among the densest serotonin innervations in brain. As determined at the electron microscopic level following immunolabelling for serotonin, virtually all serotonin varicosities in the pars reticulata and 50% of those in the pars compacta formed a synapse, essentially with dendrites. The combination of serotonin immunocytochemistry with tyrosine hydroxylase immunolabelling of dopamine neurons reveals that 20% of the serotonin synaptic contacts in the pars reticulata are on dopamine dendrites and 60% are on a type of unlabelled dendrite characterized by its peculiarly high cytoplasmic content of microtubules. The comparison of the diameter of the dendritic profiles that were in synaptic contact with serotonin-immunoreactive varicosities with the diameter of all other dendritic profiles of the same type suggests that serotoninergic varicosities innervate dopamine dendrites uniformly along their length, whereas they tend to contact microtubule-filled dendrites in more proximal regions and the other, unidentified dendrites in more distal regions. Furthermore, the size of the serotonin-immunoreactive varicosities and of their synaptic junctions is significantly smaller on dopamine dendrites and larger on microtubule-filled dendrites than on other, unidentified dendrites, indicating that the nature of the postsynaptic target is an important determinant of synaptic dimensions. These data should help to clarify the role of serotonin in the nigral control of motor functions. They indicate that this dense serotonin input to the substantia nigra is very precisely organized, acting through both “non-junctional” and “junctional” modes of neurotransmission in the pars compacta, which projects to the neostriatum and the limbic system, whereas the predominant mode of serotonin transmission appears to be of the “junctional” type in the pars reticulata, where serotonin can finely control the motor output of the basal ganglia by acting on the GABA projection neurons either directly or through the local release of dopamine by dopaminergic dendrites. The data also raise the possibility that the postsynaptic targets have trophic retrograde influences on serotoninergic terminals. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: serotonin innervation, substantia nigra, dopamine neurons, dark neurons, ultrastructure, cellular interactions.

Brain regions involved in the control of motor functions are currently known to be densely innervated by serotonin (5-HT) fibres, supporting an important role of 5-HT in the regulation of such functions. In the substantia nigra (SN), whose 5-HT input is topographically organized and originates in the mesencephalic (dorsal and median) raphe nuclei (see Ref. 10), unilateral injection of 5-HT induces a contralateral rotational behaviour, particularly in 5,7-dihydroxytryptamine-lesioned rats, supporting a role for nigral 5-HT in motor control. Although this effect might be at least partly independent of dopamine (DA), as assessed following 6-hydroxydopamine destruction of the nigral DA neurons, 5-HT excites these neurons and
induces the local dendritic release of DA, possibly through the facilitation of a calcium conductance in DA dendrites. As a morphological support, direct synaptic contacts have indeed been demonstrated between 5-HT-immunoreactive axonal varicosities, or axonal varicosities labelled following tract tracer injections into the dorsal raphe, and nigral DA [tyrosine hydroxylase (TH)-immunostained] dendrites. There is, however, no quantitative data on the cellular relationships of these terminals with either their DA targets or other types of nigral neurons. Furthermore, 5-HT varicosities have not been investigated for their synaptic incidence in the SN, as opposed to various other regions of the brain, where they have been reported to mostly lack the junctional specializations typical of synaptic contacts (for review, see Ref. 44).

The present study was undertaken to deepen our understanding of the position occupied by the 5-HT projection in the neural circuitry of the SN by providing quantitative data on the density of this input, on the proportion of 5-HT varicosities involved in morphologically-defined synapses and on their postsynaptic elements. For this purpose, we investigated separately the two parts of the SN, namely its pars compacta (SNc), which includes most of the nigral DA neurons, and its pars reticulata (SNr), which receives dendritic projections from the DA neurons of the ventral tier of the SNc and is populated by the GABA output neurons, The density of 5-HT innervation was quantified on light microscopy following immunostaining for both 5-HT and TH, using a monoclonal antibody recognizing a 5-HT-glutaraldehyde-protein conjugate;25 polyclonal antibodies against TH (Pel-Freez, Rogers, AR; a biotinylated horse anti-mouse antibody (Vector, Burlingame, CA); an avidin–biotin–peroxidase complex (ABC kit, Vectastain; Vector); goat anti-rabbit antibodies conjugated to 1 nm gold particles (AuroProbe One GAR, 1/50; Amersham, Buckinghamshire, U.K.) and a silver enhancement kit (Intense M, Amersham); pioloform (Polaron, Bio-Rad, Cambridge, MA).

Animals
The study was performed on 17 adult female Sprague-Dawley rats (225–250 g; Charles River, Montréal, QC, Canada). All efforts were made to minimize animal suffering and to reduce the number of animals used. These experiments did not involve in vivo techniques. The animals were deeply anaesthetized with sodium pentobarbital and perfused through the ascending aorta either with 300 ml ice-cold artificial cerebrospinal fluid (see Ref. 17), for [H]5-HT uptake/storage and autoradiography (n=3), or with saline buffered with 0.01 M sodium phosphate (PBS; pH 7.4) followed by 500–700 ml of 3.5% glutaraldehyde prepared in 0.1 M sodium phosphate buffer (PB, pH 7.4) containing 0.2% sodium metabisulphite, for 5-HT and TH immunocytochemistry (n=14).

Quantification of serotoninergic terminals
Slices of fresh midbrain tissue from three rats were processed for [H]5-HT uptake/storage and autoradiography in order to quantify the number of 5-HT axonal varicosities/mm of the SNc and SNr. The animals were separately fixed with glutaraldehyde and with osmium tetroxide vapours and embedded in Epon 812. From each rat, three to four semithin sections (4-μm-thick) of the whole midbrain slices were cut on a Polycut microtome (Reichert-Jung, Vienna, Austria) and dipped in liquified nuclear emulsion, which was developed in freshly prepared D-19 after three weeks exposure. Silver grain clusters representing labelled 5-HT axonal varicosities were counted by computerized image analysis (ImageSet; DappleSystems, Sunnyvale, CA). On each section, counting was performed in three regions of the SNr and two regions of the SNc, with a counting window of the size illustrated in Fig. 1. The raw data expressed as number of silver grain clusters/unit area were extrapolated to number/unit volume as described previously. For that purpose, a separate series of semithin sections (5-μm-thick) was dipped in nuclear emulsion and developed after 5-60 days to determine the effect of autoradiographic exposure time on the number of detected 5-HT axonal varicosities.

Pre-embedding immunocytochemistry
Single immunolabelling, 50-μm-thick Vibratome sections of the glutaraldehyde-fixed midbrain were rinsed in 0.05 M Tris–HCl buffer containing 0.9% sodium metabisulphite (TMBS; pH 7.4) and incubated in TMBS containing 0.5% sodium borohydride for 5–10 min. They were cryoprotected in TMBS containing 31% sucrose and 13% glycerol (15 min), frozen in liquid nitrogen-cooled isopentane and then in liquid nitrogen (10 s each) and thawed in TMBS. They were then immunostained and stained according to a procedure described elsewhere. Following a procedure described elsewhere, a biotinylated horse anti-mouse IgG (1/500) and a silver enhancement kit (Intense M, Amersham) were incubated simultaneously.
with a polyclonal antibody against TH (1/5000) and the monoclonal antibody against 5-HT–glutaraldehyde–protein conjugate for 48 h. Immunostaining for 5-HT was obtained as above and TH immunoreactivity was revealed by the immunogold procedure, using goat anti-rabbit antibodies conjugated to 1 nm gold particles which were intensified by silver enhancement. Control sections were incubated in parallel, following the same procedure, except that one of the primary antibodies was omitted. These control sections confirmed the specificity of labelling: no DAB-labelled terminals and no immunogold-labelled somata or dendrites were seen when the 5-HT or TH antibody was omitted, respectively. Moreover, previous experiments with the same antibodies, in the same conditions, on sections from 5,7-dihydroxytryptamine- or 6-hydroxydopamine-lesioned rats showed markedly reduced numbers of 5-HT- or TH-immunopositive cell bodies in the dorsal raphe or SN, respectively.32,33 In these animals, there was also a concomitant reduction of 5-HT- or TH-labelled axonal fibres in the projection areas, e.g., the SN or striatum, respectively. The immunostained sections were post-fixed with 1% osmium tetroxide in 0.1 M PB for 30 min, dehydrated in graded series of dilutions of ethanol and flat-embedded in Durcupan. After re-embedding of selected areas, serial ultrathin sections (silver, 50 nm) from the SNr and SNc were cut on an Ultratome ultramicrotome (Reichert-Jung) and collected on single-slot copper grids coated with pioloform. Sections were counterstained with lead citrate41 and examined with a Phillips EM 300 G or CM 100 electron microscopes at a working magnification of ×14,500.

Electron microscopy

To determine the synaptic incidence of 5-HT varicosities in the SNr and SNc, 5-HT-immunoreactive profiles were photographed at random in single ultrathin sections [from 10 rats for the SNr (64 ± 28/rat) and from five rats for the SNc (42 ± 29/rat)]. The area, the long and short axes and the length of synaptic contacts of the 5-HT varicosities were measured by computerized image analysis (Image 1.38; courtesy of W. Rasband, NIH) on pictures of such varicosities randomly chosen from the same material (n=366 in SNr; n=66 in SNc). The mean diameter and the aspect ratio were derived from the long and short axes: Mean diameter=(Long axis+Short axis)/2. Aspect ratio=Long axis/Short axis. The proportion of 5-HT axonal varicosity profiles showing a synaptic specialization in single thin sections (parallel, thickened membranes separated by an enlarged cleft filled with electron-dense material) was extrapolated to estimate the synaptic incidence of complete varicosities, using the stereological formula of Beaudet and Sofolo.34 The latter calculations were performed using the long axis of the varicosity profiles, as best estimate of the diameter (or height across the sectioning plane) of whole varicosities (see Ref. 54).

To see whether TH-immunopositive or microtubule-filled, electron-dense dendrites (MT; see below) were preferred synaptic targets of the innervation by 5-HT-positive terminals, we counted the number of TH-immunolabelled, profiles that were in synaptic contact with 5-HT varicosities in synaptic contact with 5-HT terminals; MT; and unidentified (unlabelled and not MT) dendritic profiles were in synaptic contact with 5-HT varicosities in the SNr. The total number of TH-immunolabelled, MT; and unidentified dendritic profiles in the same sections was also counted. The following ratios were then calculated: TH-immunolabelled dendritic profiles in synaptic contact with 5-HT terminals/total number of dendritic profiles in synaptic contact with 5-HT terminals; MT dendritic profiles/total number of dendritic profiles; MT dendritic profiles/total number of dendritic profiles of each type (TH, MT or “unidentified”) that were postsynaptic to 5-HT-positive varicosities was also measured using the image analysis system as well as that of all other dendritic profiles of the same types. These measurements and calculations were carried out on four rats (n=60 ± 12 profiles postsynaptic to 5-HT-positive varicosities and 433 ± 118 profiles that did not have 5-HT-positive synaptic contact) each.

Statistical analyses

A factorial ANOVA (Statview 4.01; Abacus Concepts, Berkeley, CA) was used to compare the number of varicosities labelled with [3H]5-HT in autoradiographs and counted in the SNr and SNc, the synaptic incidence of 5-HT-immunoreactive varicosities in the SNr and SNc, and the morphometric data on 5-HT-positive varicosity profiles that were either non-synaptic or in synaptic contact with TH-immunoreactive, MT or unidentified dendrites in the SNr. Comparison of the morphometric data on 5-HT-immunolabelled varicosities between the SNr and SNc was made using a nested ANOVA for samples of unequal sizes.35 Differences in diameter between dendritic profiles that were postsynaptic to 5-HT-immunostained varicosities and those that were not were evaluated for each type of dendrite (unidentified, TH-immunopositive or MT) using a Student’s t-test. The analysis of deviance was used to compare the synaptic incidence of 5-HT-immunolabelled varicosity profiles between the SNr and SNc and the proportions of TH-immunopositive or MT dendrites that were synaptically contacted by 5-HT-positive varicosities with the proportions of TH-immunolabelled or MT dendrites in the overall population of dendrites.36 To respect the conditions of application of the ANOVA and Student’s t-tests, we used the square root of the area of the 5-HT-immunostained varicosity profiles, the natural logarithm of the width of their dendritic targets, and the aspect ratio was transformed into arcsin 57. A posteriori tests were applied to locate differences between individual groups (ANOVA).

RESULTS

Quantitative autoradiographic estimates of serotonergic innervation

Following the uptake and storage of [3H]5-HT, light microscope autoradiographs displayed numerous silver grain aggregates, or clusters, over a background of diffuse silver grains (Fig. 1). Such silver grain clusters have previously been shown to represent labelled 5-HT axonal varicosities in the present conditions of incubation.18 This selective labelling was present in all areas of the midbrain sections but was much more prominent in the substantia nigra, particularly the SNr (Fig. 1B).

In the series of autoradiographs exposed for different periods of time, the number of silver grain clusters increased with the duration of exposure, reaching a plateau around 60 days (Fig. 2). Since the background also increased with exposure time, quantification was performed on sections exposed for 21 days, which gave an optimal signal-to-noise ratio (Fig. 1C–F), and the data were extrapolated using the curve in Fig. 2A, B.17,18 This analysis gave values of 8 x 10³ silver grain clusters/mm² in the SNr and 6 x 10²/mm² in the SNc (Table 1).

These values were extrapolated to volume using the mean diameter of the 5-HT-immunoreactive...
Fig. 1. Light micrographs of the rat SN. (A) Dopamine neurons visualized by TH immunostaining. The cell bodies are mostly concentrated in the SNC whereas many "apical" dendrites project into the SNr. (B) Semithin section of the SN showing 5-HT axonal varicosities revealed by autoradiography following \(^{3}H\)5-HT uptake/storage in vitro. The frame represents the size of the image analysis measuring window, corresponding approximately to the area of the fields illustrated in C-F. When necessary, manual adjustments of the counting window were done in order to remain within the limits of the SNC. (C, D) 5-HT axonal varicosities visualized at higher magnification as clusters of silver grains in the SNr and SNC, respectively. (E, F) Binary images of C and D, respectively, after grey level selection of the silver grain clusters. The individual features were counted by the image analysis system. Empirically determined correction factors were used to take into account the fusion of silver grain clusters into single binary image features (see Ref. 17). Scale bars=500 \( \mu m \) (A, B) and 250 \( \mu m \) (C, D).
would theoretically have been counted if all labelled 5-HT
cal. This curve served to estimate the theoretical maximum
Table
The insert shows the original data plotted as double recipro­
to transform the numbers obtained after counting in auto­
time and the silver grain clusters counted in these sections
percentages represented in this graph. This curve was used
for autoradiographic exposure time. Six series of semi thin sec­
tions were exposed for increasing periods of
varicosities, as measured by electron microscopy in the SN (Table 2), and corrected for the incomplete
detection of tritium β particles from sections more than 2.0 μm thick (see Refs 17 and 18). The extrapo­
lated values were $9 \times 10^6$ 5-HT varicosities/mm$^3$ in
$8.39 \pm 1.59$ 5.88 ± 1.34***
$	imes 10^9$/mm$^3$ (± S.D.)
$8.77 \pm 1.66$ 6.14 ± 1.40

Table 1. Density of serotonin innervation in the pars

electron micrographs from single immunostained

Ultrastructural features of serotonin-immunoreactive
varicosities in the pars reticulata and pars compacta

Light microscopic observation revealed a dense
network of fine 5-HT-immunostained varicose fibres
in the SN, particularly in the SNr. Varicosities of various sizes were observed on individual fibres
(Fig. 3).

In the electron microscope (Fig. 4), the profiles of 5-HT-immunostained varicosities were recognized by the presence of the typical diffuse electron-dense peroxidase reaction product associated with the membranes of synaptic vesicles and mitochondria and the inner surface of the plasma membrane. Consistent with previous descriptions, they contained small clear vesicles, occasional large dense core vesicles and mitochondria. In both SNr and SNC, the 5-HT-stained varicosities were ovoid in shape, which was reflected in the aspect ratio of 1.7. There was no significant difference in size between 5-HT-positive varicosities of these two regions (Table 2).

In most cases, the 5-HT-immunostained profiles did not show any synaptic membrane specialization when examined in single ultrathin sections (Fig. 4A, B). When present, the synapse was generally of the asymmetrical type (Figs 4D, 5B), as reported previously but see Ref. 31). The major difference between the two regions of the SN concerned the frequency of synaptic contacts (synaptic incidence) displayed by 5-HT-positive profiles, which was twice as great in the SNr as in the SNC (Table 3). Extrapola­
tion to estimate the synaptic incidence for complete 5-HT varicosities showed that the large majority, if not all, were synaptic in the SNr, whereas only half were synaptic in the SNC (Table 3).

Synaptic targets of serotonin-immunoreactive varicosities

Serotonin-immunoreactive varicosities made
synaptic contacts predominantly with dendritic shafts
ranging from 0.4 to 1.6 μm in diameter (Figs 4C, D and 6). In the SNr, 5-HT-immunostained varicosities
Table 3. Synaptic incidence of serotonin-immunoreactive axonal varicosities in the substantia nigra pars reticulata and compacta

<table>
<thead>
<tr>
<th></th>
<th>SNr</th>
<th>SNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of varicosities</td>
<td>593</td>
<td>217</td>
</tr>
<tr>
<td>Synaptic incidence in single thin sections (%)</td>
<td>28 ± 8</td>
<td>13 ± 6***</td>
</tr>
<tr>
<td>Extrapolation to whole varicosities (%)</td>
<td>93</td>
<td>49</td>
</tr>
</tbody>
</table>

Serotonin-immunostained axonal varicosities were photographed at random in single ultrathin sections of the SNr and SNC from 10 and six rats, respectively. The proportion of 5-HT-positive profiles showing a synaptic specialization was determined and then extrapolated to whole varicosities, according to the stereological formula of Beaudet and Sotelo, using the long axis as an estimation of the diameter of the whole varicosities.* * *(***P<0.001, Deviance analysis.)

made contact together with unlabelled varicosities to form "en rosette" arrangements around dendritic profiles in cross-section. The length of the synaptic contacts ranged between 0.03 and 0.75 µm and varied depending on the target type (see below). No example of synaptic contacts by 5-HT-positive varicosities on perikarya was observed in the SNC or in the SNr.

In dual immunostained sections, the DAB reaction product for 5-HT was detected at a greater depth in the Vibratome slices than the silver-intensified immunogold labelling for TH. In ultrathin sections from the superficial region of the slices, 5-HT-immunoreactive varicosities in the SNr were frequently observed in synaptic contact with TH-immunostained dendrites (Fig. 5A, B). TH-immunolabelled dendrites represented 20 ± 5% of the structures postsynaptic to 5-HT-positive terminals, whereas they represented only 13 ± 4% of the total number of dendritic profiles counted in the same sections of the SNC. The difference between these percentages was statistically significant (P<0.05), indicating a slight over-representation of TH-positive dendrites as postsynaptic targets of the 5-HT innervation. The diameter of the TH-immunoreactive dendritic profiles ranged from 0.45 to 2.26 µm (mean ± S.D.=1.06 ± 0.46 µm). The diameter of those that were postsynaptic to 5-HT varicosities was similar, ranging from 0.34 to 2.23 µm (1.06±0.57 µm) (Fig. 6B).

Among the structures postsynaptic to 5-HT-positive terminals, TH-immunoreactive profiles had a significantly larger diameter than the unlabelled, unidentified dendritic profiles (P<0.05). The latter (Fig. 6A) ranged between 0.20 and 2.20 µm (0.84±0.52 µm). The unidentified dendritic profiles that were not postsynaptic to 5-HT varicosities were significantly larger than those that were postsynaptic, ranging between 0.30 and 4.30 µm (1.54±0.89 µm; P<0.001), indicating that 5-HT-positive varicosities preferentially contacted these dendrites in their distal regions, unless a subpopulation of unidentified neurons, with larger dendrites, is not synaptically contacted by the 5-HT innervation.

Some of the dendrites, unlabelled in TH-immunostained sections, were characterized by the presence of numerous microtubules (Fig. 4D). The cytoplasmic electron density of these dendrites (MT) ranged from clear to very dense, the one illustrated in Fig. 4D being intermediate in this respect. The contour of their cytoplasmic membrane was also often irregular. They represented 5 ± 2% of the total number of dendritic profiles in the SNr and they
Fig. 4. Electron micrographs of 5-HT-immunoreactive axonal varicosities in the SN. (A, B) Axonal varicosities of the SNc that do not display junctional membrane specializations. Both are surrounded by unlabelled axons. (C) 5-HT-immunopositive axonal varicosity in synaptic contact with an unlabelled dendrite in the SNr. Several unlabelled terminals are also in synaptic contact with the same dendrite, forming a typical "en rosette" arrangement. (D) 5-HT-positive axonal varicosity forming an asymmetrical synaptic contact with a dendrite in the SNr characterized by a peculiarly high number of microtubules (MT), here in cross-section. Note the presence of some vacuoles and the slightly irregular contour of the cytoplasmic membrane of this profile, which also appears lightly electron-dense between the microtubules. Scale bar=0.5 µm (A–D).
Fig. 5. (A, B) 5-HT axonal varicosities (5-HT-immunoreactive; diaminobenzidine reaction product) in synaptic contact (arrows) with dopamine dendrites (TH-immunoreactive; silver-intensified immunogold particles) following dual pre-embedding immunocytochemistry. The synapse in B is clearly asymmetrical, as was the majority of synapses formed by 5-HT-immunoreactive varicosities in the substantia nigra. Scale bar=0.5 μm (A, B).

represented 6±2% of all postsynaptic targets of the 5-HT innervation. The diameter of the MT profiles that were postsynaptic to 5-HT-positive terminals was not statistically different from the TH-immunoreactive or the unidentified dendritic targets of 5-HT-immunoreactive axon terminals. They were, however, significantly larger than the MT dendritic profiles that were not contacted by 5-HT-positive varicosities (Fig. 6C), which suggests that the MT dendrites were preferentially innervated by 5-HT-positive terminals in their proximal portion.

Morphometrics of serotonin-containing varicosities in contact with different dendritic types

The area, diameter, aspect ratio and length of the junctional complex of the 5-HT-positive varicosity profiles that were non-synaptic or were in synaptic contact with unidentified, TH-immunoreactive or MT dendrites are shown in Table 4. Non-junctional 5-HT-positive varicosity profiles were significantly smaller than synaptic ones contacting unidentified or MT dendrites, but comparable to those contacting
The results of the present study provide new information concerning the 5-HT innervation of the substantia nigra (i.e. \[^3^H\]5-HT-labelled or 5-HT-immunoreactive axonal varicosities). First, they point to the fact that both the SNc and SNr receive a dense 5-HT input and that the density of innervation is greater in the SNr than in the SNc, exceeding that of any region of the brain so far examined. Secondly, they demonstrate that, unlike most other regions of the brain, virtually all 5-HT varicosities form synaptic specializations in the SNr, whereas only 50% do so in the SNc. Thirdly, they identify in the SNr both DA and non-DA dendrites, among which is a hitherto unidentified category characterized by a high density of microtubules, as synaptic targets for 5-HT terminals. Finally, they demonstrate that the morphometrics of 5-HT synaptic boutons in the SNr are related to their synaptic targets.

Density of serotonin innervation in the substantia nigra

Our quantitative results reveal that the SN receives one of the densest 5-HT projections in the brain (6–9 millions of varicosities/mm\(^3\)). They are consistent with previous reports having shown that the ventral mesencephalic tegmentum including the SN contains the highest brain concentrations of endogenous 5-HT and the highest density of binding sites for high-affinity ligands of the 5-HT transporter.\(^{15,16,20,38}\) For comparison with other basal ganglia structures, the mean density of 5-HT innervation was previously estimated to be \(4.5 \times 10^6\) varicosities/mm\(^3\) in the globus pallidus, \(3 \times 10^6\) varicosities/mm\(^3\) in the nucleus accumbens and \(2.6 \times 10^6\) varicosities/mm\(^3\) in the neostriatum.\(^{56}\) The density of 5-HT innervation in the normal neostriatum of adult rat was recently re-examined in the conditions used for the present experiments, which decrease background labelling resulting from low affinity uptake in DA terminals (see Ref. 17); it was estimated to be \(5.4 \times 10^6\) varicosities/mm\(^3\).\(^{48}\)

The fact that the SNr contains a significantly higher density of 5-HT varicosities than the SNc is also in agreement with early biochemical measurements,\(^{49}\) as well as with immunohistochemical descriptions in the rat\(^{27,49}\) and in the monkey.\(^{29}\) Other studies based on anterograde tracing from the dorsal raphe nucleus, however, showed that the majority of the raphe-nigral afferents innervate the SNc.\(^{2,22,57}\) Considering the topographical organization of this projection,\(^{10}\) these results were presumably biased by a preferential labelling of 5-HT neurons projecting to the SNc. They could also have involved a substantial number of non-5-HT neurons which represent more than two thirds of the neuronal population in the dorsal raphe nucleus.\(^{14}\)

Synaptic innervation of the substantia nigra

The present electron microscopic data indicate that the 5-HT innervation is entirely synaptic in the SNr and only 50% synaptic in the SNc, where the density of innervation is also lower. It has been suggested that two morphologically distinct 5-HT fibre systems, varicose fibres and beaded fibres, innervate some forebrain regions with different synaptic incidences.\(^{51}\)
However, in spite of a 100% synaptic incidence, we observed both small and large boutons in the SNr. Moreover, both types of boutons could even be present on the same parent axon, as observed in the light microscope. This indicates that both types of 5-HT varicosities form synapt contacts in that part of the SN at least.

The lower synaptic incidence of 5-HT varicosities in the SNC supports the idea that 5-HT, in addition to acting at synaptic specializations, also acts at a distance by diffusion in the extracellular space, as initially proposed for 5-HT in the cerebral cortex (see Ref. 11), to reach 5-HT receptors on the DA neurons. Interestingly, a synaptic incidence of 50% has also been calculated for 5-HT terminals in the VTA,26 which may represent a continuum of the dorsal SNC.

In contrast, the “junctional” type of transmission appears to be the major mode of action for 5-HT in the SNr. This region comprises the GABA output neurons of the basal ganglia and the “apical” dendrites of the “inverted pyramidal” SNC DA neurons.21 It remains to be established whether the 5-HT varicosities which form synaptic contacts in the SNC also contain a high content of microtubules (MT) and therefore, non-identified dendrites that could belong to more than one neuronal type. The fact that these 5-HT varicosities exhibited different terminal features indicates that they probably belong to different neuronal populations, while confirming that DA neurons are not the sole synaptic targets of the 5-HT innervation: TH-immunoreactive (DA) dendrites, which appeared to be somewhat over-represented and could therefore constitute preferential targets, unlabelled dendrites characterized by a high content of microtubules (MT) and therefore, non-identified dendrites that could belong to more than one neuronal type. The fact that these three types of dendrites exhibited different morphometric features indicates that they probably belong to different neuronal populations, while confirming that DA neurons are not the sole synaptic targets of the 5-HT innervation in the SNC.21 This provides an anatomical substrate for the in vivo locomotor effects of locally applied 5-HT agonists after destruction of DA neurons with 6-hydroxydopamine.36 Among non-DA targets, GABAergic output neurons are good candidates since 5-HT was reported to have an excitatory action, via 5-HT2C receptors, on SNr neurons.43 The MT dendrites, whose high content in microtubules would reflect a peculiar cytoskeleton organization, have not been described previously. Preliminary observations following post-embedding immunocytochemistry suggest that they may be GABAergic (unpublished observations), but whether they belong to projection neurons and/or to interneurons remains to be determined. The electron density and irregular contour of the membrane of some of these profiles suggest that they might represent so-called “dark neurons”,6 an artifact produced

### Table 4. Morphometric features of serotonin-immunoreactive axonal varicosity profiles that were non-synaptic or contacting different dendritic targets in the substantia nigra pars reticulata

<table>
<thead>
<tr>
<th>Postsynaptic targets</th>
<th>(Non-synaptic)</th>
<th>TH dendrites</th>
<th>Unidentified dendrites</th>
<th>MT dendrites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>145</td>
<td>42</td>
<td>164</td>
<td>15</td>
</tr>
<tr>
<td>Area (µm²)</td>
<td>0.29±0.16</td>
<td>0.25±0.17</td>
<td>0.35±0.19</td>
<td>0.52±0.21</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>0.61±0.18</td>
<td>0.55±0.20</td>
<td>0.67±0.19</td>
<td>0.83±0.17</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>1.7±0.6</td>
<td>1.7±0.5</td>
<td>1.7±0.6</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>Junctional complex</td>
<td>0.26±0.13</td>
<td>0.31±0.13</td>
<td>0.38±0.14</td>
<td></td>
</tr>
</tbody>
</table>

5-HT-positive varicosities and junctional complexes were measured in single thin sections of 5-HT/TH dually-immunostained Vibratome sections. *not statistically different, **P<0.01, ***P<0.001, factorial ANOVA and Scheffe F procedure for post hoc comparisons.
varicosities of different sizes were present on the same parent 5-HT axons, this observation raises the possibility that the size of the 5-HT varicosities could be partly dependent on local interactions with their postsynaptic targets. A retrograde effect of the target region as a whole on the size of afferent terminals has indeed been reported by Zwimpfer et al., 59 following experiments with peripheral nerve transplants inducing ectopic growth of optic nerve axons into the cerebellum; the authors suggested a role for neurotrophins in these effects. Infusions of nerve growth factor have also been demonstrated to induce a hypertrophy of cholinergic terminals in the lesioned cerebral cortex.54

CONCLUSIONS

Serotonin neurons are generally viewed as neurons with diffuse and widespread projections bearing few synaptic specializations1,2,3,4,5 and therefore involved mainly in a mode of communication also referred to as “volume transmission”, as opposed to “wiring transmission”.1,2,3 The present data indicate that both “non-junctional” and “junctional” transmission occur in the SNr, whereas the predominant mode of transmission in the SNr is apparently of the “junctional” type. Considering the high density of the nigral 5-HT input, the point-to-point cellular interrelationships established by this input in the SNr should constitute one important link in the neuronal circuitry subserving the role of the SN in the control of motor functions. The fact that the 5-HT fibres in the SNr, which also receives a topographically-organized projection from the neostriatal matrix (see Ref. 21), are in a situation not only to directly excite the GABA output neurons, but also to precisely control the dendritic release of DA in their microenvironment, appears of significant value in this respect.

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REFERENCES


*in vitro*: evidence for a direct action mediated by 5-hydroxytryptamine \( \text{SR} \) receptors. *Neuroscience* 69, 903–913.


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