Tectonigral Projections in the Primate: A Pathway for Pre-Attentive Sensory Input to Midbrain Dopaminergic Neurons

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Abstract

Much of the evidence linking the short-latency phasic signaling of midbrain dopaminergic neurons with reward-prediction errors used in learning and habit formation comes from recording the visual responses of monkey dopaminergic neurons. However, the information encoded by dopaminergic neuron activity is constrained by the qualities of the afferent visual signals made available to these cells. Recent evidence from rats and cats indicates the primary source of this visual input originates subcortically, via a direct tectonigral projection. The present anatomical study sought to establish whether a direct tectonigral projection is a significant feature of the primate brain. Injections of anterograde tracers into the superior colliculus of macaque monkeys labelled terminal arbors throughout the substantia nigra, with the densest terminations in the dorsal tier. Labelled boutons were found in close association (possibly indicative of synaptic contact) with ventral midbrain neurons staining positively for the dopaminergic marker tyrosine hydroxylase. Injections of retrograde tracer confined to the macaque substantia nigra retrogradely labelled small to medium sized neurons in the intermediate and deep layers of the superior colliculus. Together, these data indicate that a direct tectonigral projection is also a feature of the monkey brain, and therefore likely to have been conserved throughout mammalian evolution. Insofar as the superior colliculus is configured to detect unpredicted, biologically salient, sensory events, it may be safer to regard the phasic responses of midbrain dopaminergic neurons as 'sensory prediction errors' rather than 'reward prediction errors', in which case, dopamine-based theories of reinforcement learning will require revision.

Keywords

basal ganglia; oculomotor; dopamine; reward; superior colliculus

Introduction

The short-latency phasic response of dopaminergic (DA) neurons is considered critical for reinforcement learning (Dayan & Balleine, 2002; Schultz, 2002; Montague *et al.*, 2004).

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Typically, midbrain DA neurons exhibit stereotyped, short latency (<100 ms), short duration (~100 ms) population responses to unpredicted, biologically salient events (Schultz, 1998). Many believe this signal provides the distributed reinforcement learning mechanism in the basal ganglia and frontal cortex with a 'reward prediction error' that is used to adjust subsequent behavioral selections in a manner which maximises future acquisitions of reward (Dayan & Balleine, 2002; Schultz, 2002; Montague *et al.*, 2004).

Despite considerable support for the reward prediction error hypothesis (Schultz, 2006), there are significant observations that conflict with it (Redgrave *et al.*, 1999; Redgrave & Gurney, 2006; Redgrave *et al.*, 2007). First, phasic DA responses are not restricted to reward-related events (Horvitz, 2000; Takikawa *et al.*, 2004). Second, these responses are often remarkably stereotyped across species, stimulus modalities, and experimental paradigms (Schultz, 1998). Third, comparison of the latencies of DA neuron responses (Schultz, 1998) to gaze-shift latencies (Munoz & Guitton, 1986; Jay & Sparks, 1987) indicates that phasic signalling is conducted on the basis of pre-attentive sensory processing occurring before foveation. This is important because the information in the short-latency input to DA neurons must be constrained by the perceptual processing capacity of the sensory systems delivering this information. It is therefore crucial to determine the source(s) of short latency sensory activity in ventral midbrain DA neurons (Kitai *et al.*, 1999; Pan & Hyland, 2005). We have discovered a previously unreported tectonigral pathway in the rat and cat (Comoli *et al.*, 2003; McHaffie *et al.*, 2006), and have demonstrated in the rat that this subcortical projection via the superior colliculus (SC) provides the primary, if not exclusive, source of short latency visual input to midbrain DA neurons (Dommett *et al.*, 2005).

However, much of the evidence linking phasic DA signalling to reward-prediction errors comes from recording visual responses in DA neurons of monkeys (Morris *et al.*, 2004; Takikawa *et al.*, 2004; Bayer & Glimcher, 2005; Schultz, 2006) due to their expected similarity to humans. Thus, it is critical to establish whether the SC is a potential source of short-latency visual input to DA neurons in monkeys. Moreover, basal ganglia systems in primates may be more complex than or exhibit differences from other species (Haber *et al.*, 1995; Damier *et al.*, 1999; Graybiel, 2001; Frankle *et al.*, 2006; Sakai et al., 2002). Furthermore, monkey DA cells are divided into two tiers: the calbindin-positive dorsal tier, which receives input primarily from the ventral striatum, with minor inputs from amygdala and cortex; and the calbindin-negative ventral tier, which receives input primarily from dorsal striatum, but no input from cortex or amygdala (Lynd-Balta & Haber, 1994; Haber & Fudge, 1997). We wished to determine whether the tectonigral projection would also exhibit tier specificity in primates. Consequently, we have employed both anterograde and retrograde tracers to establish the presence and targets of a macaque tectonigral projection (May *et al.*, 2006).

Materials and Methods

All animal protocols used in this study were approved by the appropriate Institutional Animal Care and Use Committees, and were undertaken in compliance with the Guide for the Care and Use of Laboratory Animals at AALAC approved facilities. For the anterograde tracing experiments, a total of 9 adult and young adult macaque monkeys (*M. fascicularis and mulatta*) of both sexes were used; 5 received injections of biotinylated dextran amine (BDA), and 4 injections of *Phaseolus vulgaris* leucoagglutinin (PhaL). For the retrograde tracing experiments, wheat germ agglutinin conjugated horseradish peroxidase (WGA-HRP) was used as the tracer. Five relevant cases were selected for analysis from the library of *Macaca nemestrina* tissue kept in the laboratory of Prof. Suzanne Haber. Consequently, no further animals were used for this part of the investigation.

Anterograde experiments

Tracer injections into the superior colliculus—The animals were subdued with ketamine HCl (10 mg/kg, IM), supplied with an intravenous line and a endotracheal tube, and anesthetized with isoflurane (1.5-3.0 %). Carprophen (3 mg/kg, IM) was given as a presurgical anesthetic. During the surgery, the animals were hydrated with a lactated Ringer's drip, and given dexamethasone (2.5 mg/kg, IM) to control edema and atropine sulfate (0.05 mg/kg, IM) to limit secretions. Core body temperature, heart rate, and exhaled gasses were recorded and maintained within normal parameters. These animals were placed in a stereotaxic head holder and the scalp incised along the midline. Following a craniotomy, the cortex over the midbrain was aspirated to reveal the midbrain surface. A 1.0 μl Hamilton syringe held in a micromanipulator at an angle of 20°, tip up in the parasagittal plane, was used to inject BDA (Moleular Probes, Eugene, OR, USA) into the exposed SC. The 10 % BDA solution (0.1-0.2 μl) was pressure injected at 2-3 sites located 1.5 mm beneath the collicular surface. Alternatively, iontophoresis was used to inject PhaL (Vector Laboratories, Burlingame, CA, USA) into the SC. A positive current (0.7 μA, 10-20 min, 50 % duty cycle, 7 sec each) was used to expel the PhaL (4 % solution in 0.1 M, pH 8.0 phosphate buffer (PB)) from a glass micropipette (tip diameter of 20-30 μm) inserted into the SC. Following these injections, the aspiration defect was filled with damp gelfoam, and the scalp was closed with suture. The incision was infused with Sensorcaine, and the animal was given Buprenex (0.01 mg/kg, IM) as a postoperative analgesic.

Animals injected with BDA survived for ~ 21 days, and animals injected with PhaL survived for ~14 days, to allow the tracer to transport. They were then sedated with ketamine HCL (10 mg/kg, IM), and deeply anesthetized with sodium pentobarbital (50-70 mg/kg, IP). Once insensate, they were perfused through the heart with 0.1 M, pH 7.2 phosphate buffered saline (PBS), followed by a fixative containing 1.0 % paraformaldehyde and 1.25 % glutaraldehyde in 0.1 M, pH 7.2 PB. Animals intended for immunohistochemistry were instead perfused with 4.0 % paraformaldyde in 0.1 M, pH 7.2 PB. The brains were postfixed with the same fixative at 4° C for 1-2 hrs (1.0 % / 1.25 %) or overnight (4 %).

Immunohistochemical and histochemical procedures—Brains containing BDA injections were cut into 100 μm sections on a vibratome. Brains with PhaL injections were equilibrated in a 30 % sucrose cryoprotectant, frozen, and cut into 50 μm sections on a sliding microtome. For detailed descriptions of the techniques used see McHaffie and colleagues (McHaffie *et al.*, 2006). Briefly, each ordered series of sections containing BDA was incubated over night at 4° C in a 1:5000 solution of avidin conjugated to horseradish peroxidase (avidin-HRP)(Vector laboratories, Burlingame, CA, USA) in 0.1 M, pH 7.2 PB with 1.0 % Triton-X-100. They were then washed in PB and reacted in a solution containing 0.5 % diaminobenzidine HCl (DAB) and 0.01 % cobalt chloride and nickel ammonium sulphate, to which 0.005 % H_2O_2 was added to initiate the reaction. To reveal the PhaL, sections were immersed in a solution of 0.3 % Triton-X-100 and 10 % normal goat serum (NGS) in 0.1 M, pH 7.2 PB. They were then incubated overnight in a solution of 1: 200 biotinylated goat anti-PhaL (Vector Laboratories, Burlingame, CA, USA) in 0.1 M, pH 7.2 PB with 1 % NGS. The goat antibody was tagged with HRP by use of a goat ABC kit (Vector Laboratories, Burlingame, CA, USA). The HRP was then visualized with DAB, following the procedure outlined above, except that cobalt chloride was omitted from the reaction mixture. Following the DAB reaction, both sets of sections were rinsed, mounted on gelatinized slides and dried. The slides were then counter-stained with cresyl violet, dehydrated, cleared and cover-slipped.

To reveal the relationship of the PhaL labelled axon terminals to tyrosine-hydroxylase (TH) containing DA neurons, a two step procedure was used. First the PhaL was revealed using the procedures described above. After the sections were washed, they were incubated overnight at 4°C in mouse antibody to TH (1:3,000 dilution in 0.1 M, pH, 7.2 PB with 2 % NGS and 0.3 % Triton-X-100)(Chemicon International, Temecula, CA, USA). Sections were then washed and treated using standard procedures for the mouse ABC kit (Vector Labs, Burlingame, CA, USA). The HRP marking TH-positive (TH$^+$) cells was visualized by use of a VIP staining kit (Vector Labs, Burlingame, CA, USA). These sections were mounted onto gelatinized slides, dehydrated, cleared and cover-slipped.

Analysis—A drawing tube mounted on an Olympus BH-2 microscope was used to plot the location and illustrate the morphology of BDA and PhaL labelled axonal arbors and TH$^+$ neurons. Examples of anterograde labelling within SN were photographed using a Nikon Eclipse-600 microscope equipped with a Nikon DXM 1200 digital colour camera. Metamorph Software (Molecular Devices, Sunnydale, CA, USA) was used to acquire the digital images. Up to 20, 1 μm z-axis slices were combined by use of the stack arithmetic function to make the photomicrographs. The pictures were further adjusted with Photoshop^{TM} (Adobe, San Jose, CA, USA) software, using the color balance, brightness and contrast functions until the digital images approximated the observed image.

Retrograde experiments

Fudge and Haber (2000; 2001) have previously described both the WGA-HRP injections into the midbrain DA cell containing regions of the 5 monkeys used here, and the patterns of retrograde transport to the amygdala and bed nucleus of the stria terminalis they produced. Further details of the methodology can be found therein.

Tracer injections into the ventral midbrain—Initial sedation via ketamine HCl (10 mg/kg, IM) was followed by inducing a deep surgical level of anesthesia using Na pentobarbital (initial dose 20 mg/kg, IV, with maintenance dosing as needed). WGA-HRP (4.0%, Sigma, St. Louis, MO, USA) was pressure injected (35-40 nl) into discrete regions of the ventral midbrain by use of a 0.5 or l.0 μl Hamilton syringe. The animals were deeply anesthetized with pentobarbital 7-10 days after surgery, and underwent intracardiac perfusion with saline and 4 % paraformaldehyde, as described above.

Immunohistochemical procedures—Once the brains were cryoprotected in sucrose, serial coronal sections (50 μm) were cut on a freezing microtome, and processed to reveal the WGA-HRP by use of immunocytochemical procedures. Sections were first rinsed in 0.1 M, pH 7.4 PBS with 0.3% Triton X-100 (PBS-T), and then preincubated in 10% normal goat serum (NGS) diluted with PBS-T (NGS-PBS-T) for 30 min. Subsequently, they were placed in the primary antisera (rabbit anti-WGA-HRP, 1:2000 (Sigma, St. Louis, MO, USA)), in NGS-PBS-T for approximately 96 h at 4°C. The avidin-biotin reaction (rabbit Vectastain ABC kit, Vector Labs, Burlingame, CA, USA) was used to visualize the tracer. Tissue was incubated for 10-12 min in DAB with cobalt chloride and nickel as described above, to yield a black reaction product. In all cases, the tissue was either counterstained or adjacent sections were stained for Nissl substance. These sections were mounted onto gelatinized slides, dehydrated, cleared and coverslipped.

Analysis—The selected cases had well localised injections in the ventral midbrain that lacked significant necrosis. The locations of retrogradely labelled neurons in the SC were charted using a Nikon Eclipse 800 microscope equipped with brightfield/darkfield illumination, an imaging system, and Neurolucida software (MicroBrightfield, Williston, VT, USA). Photomicrographs were captured digitally, and all image files were processed

using Photoshop™ (Adobe, San Jose, CA, USA), Illustrator ™ (Adobe, San Jose, CA, USA), or FreeHand™ (Macromedia, San Jose, CA, USA) software. The images were corrected for contrast, brightness, and color balance. Analysis of the location of retrogradely labelled tectonigral neurons was conducted by plotting the position of each neuron in 3 representative sections selected from the rostral, central and caudal SC onto a drawing of the SC boundaries and layers using the Neurolucida software. Care was taken to select the same 3 levels from each case. Except where specifically mentioned, the number of labelled neurons quoted for each injection site refers to the summed total of labelled cell bodies plotted within the boundaries of the SC on each of the three analysed sections. These raw numbers were not corrected or statistically analyzed as they were solely intended to allow comparison of relative labelling densities with respect to injection site location.

Results

Anterograde Labelling of Tectonigral Axons

Anterogradely labelled axons and terminals were found within the SN in all 9 animals that received collicular injections of BDA or PhaL. Selected cases will be illustrated to convey qualitative descriptions of the anterograde tracing observed within this group.

Anterograde labelling with BDA—An example of a BDA injection site that was confined to the SC, and primarily located within the intermediate gray layer (SGI) of the caudal third of the tectum, is illustrated in figure 1 (insert lower right). [NB: The definition of collicular layers and the nomenclature used to describe them follow the system described in May and Porter (1992)]. In coronally sectioned material, labelled axons from this injection were found throughout the rostrocaudal extent of the ipsilateral SN (Fig. 1A-F), and were located in both pars compacta (SNc) and pars reticulata (SNr). Labelled axons in SNr were generally more numerous in the lateral aspects of the nucleus (Fig. 1B-E). Labelled axons in SNc tended to be denser dorsally, particularly at caudal levels (Fig. 1C-F). Numerous labelled axons were also present in the midbrain reticular formation immediately dorsal to SNc (Fig. 1C-E), as well as in the ventral tegmental area (VTA) medial to SNc (Fig. 1B-E). They were also observed in the subthalamic nucleus (Fig. 1A-D). A small number of BDA labelled axons were found contralaterally in the SNc, SNr and VTA (not illustrated).

Close examination of the anterogradely labelled axons in SN revealed that most were terminal arbors (Fig. 2). The BDA labelled terminal arbors observed in coronal sections through the SNc had relatively fine diameters, and displayed occasional branches (Fig. 2A-D & F). Along their course, they had small enlargements which were at either *en passant* or terminal locations. While ultrastructural verification is necessary, these presumably represent synaptic boutons. Individual axonal arbors extended among the counter-stained cells in SNc, with boutons displaying close associations (arrowheads) with several different neurons (Fig. 2A-D & F). The BDA labelled arbors usually contributed just a few boutons to any individual neuron in SNc (Fig. 3B), but in some cases multiple boutons were seen in close association (arrowheads) with a single SNc cell (Fig. 3A). These displayed a light brown color due to the presence of neuromelanin granules. BDA labelled terminals were also observed in SNr (Fig. 1A-E), and a portion of these labelled terminal arbors displayed boutons that lay in close association with nigrotectal neurons that had been retrogradely labelled with BDA (Fig. 2G-I). These BDA labelled axons often followed labelled nigrotectal dendrites, contributing a number of close boutonal associations along their course (Fig. 3C). Such close associations are suggestive of synaptic contact between the labelled elements. However, proof of actual synaptic contact requires electron microscopic verification, such as that previously shown between rodent tectonigral terminals and TH⁺

profiles (Comoli et al., 2003). In other systems it has been noted that more than half of observed close associations represent synaptic contacts (Pilowsky et al., 1992)

Anterograde labelling with PhaL—Fast spiking GABAergic output neurons of SNr have local axon collaterals that terminate both within SNr and SNc (Grofova *et al.*, 1982; Paladini *et al.*, 1999; Mailly *et al.*, 2003). Consequently, it was possible that the retrogradely transported BDA that labelled nigrotectal neurons in the cases described above might also have labelled their local axon collaterals within SN. The fact that the anterogradely labelled terminals generally appeared to contain denser label than the retrogradely labelled cells argues against this possibility. Nevertheless, to resolve any potential ambiguity associated with BDA tracing, a further 4 subjects received intracollicular injections of the anterograde tracer PhaL. While in some species, PhaL can be occasionally transported in the retrograde direction, this is rarely the case in primates. Combined immunohistochemical procedures were used to reveal the presence of PhaL labelled terminal arbors and TH^{+} neurons in the same section. Tissue was sectioned in either the coronal or parasagittal planes to further explore the trajectories of tectonigral fibers. In the illustrated example, the injection of PhaL was restricted to a narrow cylindrical area in the caudal SC, crossing all layers (Fig. 4, upper right), and the distribution of labelled fibers is shown in a medial to lateral series of parasagittal sections through the ipsilateral SN (Fig. 4A-F). Although the injection was small and labelled fewer axons than the BDA case illustrated in figure 1, the two tracers provided qualitatively similar results. Thus, most of the labelled axonal arbors were found in SNc (Fig. 4A-E), with the highest concentration in this subject located medially within the dorsal tier (Fig. 4A-C). In this case, only a few labelled axons were found in SNr (Fig. 4C&E), but PhaL labelling in SNr was more evident in other cases (Fig. 3E&F), even in the absence of retrogradely labelled nigrotectal neurons. As was the case for the BDA labelling, PhaL labelled axons were present in the reticular formation dorsal to SNc (Fig. 4A-B), as well as in the ipsilateral VTA (Fig. 3G) and subthalamic nucleus (not illustrated).

The terminal arbors labelled in the illustrated case were made up of fine axons that rarely branched, with the exception of short perpendicular extensions (Fig. 5). They were ornamented with many *en passant* and terminal boutons that formed close associations with TH^{+} neurons (Fig. 5A-D). The labelled axons could often be followed in the parasagittal plane for considerable distances through the ipsilateral SNc (Fig. 5C). Again, the typical pattern was for an individual axon to have a few boutons associate with each of several TH^{+} neurons encountered along its course (Fig. 5A-C). In contrast, the labelled axonal arbors seen in ipsilateral SNr were not associated with TH[+] somata (Fig. 5E). The photomicrographs in figure 6 show further examples of presumptive contacts formed between anterogradely labelled tectonigral terminals and TH^{+} neurons in SNc (Fig. 6A,B,E & F) and a terminal arbor in SNr (Fig. 6D). In most cases, only a few boutons were associated with each SNc cell (Fig. 6E), while in others, a cluster of boutons lay adjacent to a TH^{+} neurons (Fig 6. A&B), or the axon could be followed along a TH[+] dendrite (Fig. 6F). Occasionally, neurons with large numbers of associated boutons were present (Fig. 3H). In this case, numerous boutons sit along SNc cells, which were brown in color due to their neuromelanin content. Finally, it was observed that while PhaL labelling in SN was predominantly ipsilateral, a small number of labelled arbors with similar morphology were present contralaterally. These terminal arbors had a similar morphology to those present ipsilaterally, and were mainly distributed amongst neuromelanin containing cells in SNc (Fig. 3D).

In summary, following the use of either of two anatomical tracers, our anterograde tracing data revealed the presence of a direct tectonigral projection in the primate. The tectonigral terminal boutons targeted TH[+], presumably DA cell bodies in SNc, ending more densely in the dorsal tier. They also targeted the SNr neuropil, and perhaps even nigrotectal neurons.

The next stage of the investigation was to confirm the tectonigral projection using retrograde tracing techniques, and to provide a regional description of the cells of origin of the tectonigral pathway in the primate.

Retrograde Labelling of Tectonigral Cells

Injection sites—Tectonigral cells in the monkey SC were identified within 5 cases in which there were 9 injections of WGA-HRP into the ventral midbrain (Fig. 7). The photomicrograph in figure 7A shows an example of one injection site (Case 91L) where the uptake zone of the tracer was judged to be contained entirely within the SN. The maximum extents of the 9 injections were plotted in relation to the location of ventral midbrain DA neurons defined in adjacent sections (Fig. 7B). Each injection was classified as being either restricted to the SN or the SN and VTA (light grey shading), or predominantly involving these regions, but including dorsal spread into adjacent tissue (dark grey shading).

Retrogradely labelled tectonigral neurons—In all 5 cases, an overwhelming majority of WGA-HRP labelled neurons (93.6 ± 0.95 %) were located in the intermediate and deep layers of the SC (Fig. 8A). Morphologically, they represent a restricted subset of the different cell types previously described in the primate SC (Ma *et al.*, 1990). The 4 most common classes of retrogradely labelled cells were small neurons that were ovoid, bipolar, or pyramidal in shape, and medium-sized multipolar neurons. All these types were distributed in the intermediate gray and white, and deep gray layers of the SC (SGI, SAI and SGP, respectively). Small ovoid neurons (Fig. 8B) made up about half the labelled cells, and represented the largest class of tectonigral neurons. They had small somata (long axis ~ 10-20 μm) from which several processes extended abruptly. These ovoid neurons appeared throughout the mediolateral and rostrocaudal extent of the SC. Small bipolar neurons (Fig. 8B) made up approximately 15-20% of the labelled cells. They had elongated cell bodies (long axis ~ 25-35 μm), and two tapering dendritic processes. The long axes of these cells were more commonly aligned perpendicular to the collicular surface, than parallel to it. These bipolar neurons displayed a tendency for a more lateral distribution. Small pyramidal neurons (Fig. 8C) represented a further 15-20% of labelled cells. They had triangular shaped somata (long axis ~ 20-35 μm) with dentritic processes emerging from each point. Medium-sized, multipolar neurons (Fig. 8D) represented the remaining significant class of retrogradely labelled tectonigral neurons (~10-15%). They were a heterogeneous class, whose somata were larger (long axis ~ 30-50 μm) than those of the other tectonigral populations. Typically, their somata tapered into several dendrites giving them a piriform or stellate appearance. Notably absent from the cell types labelled with WGA-HRP were the classes of large multipolar output neurons (Norita, 1980;Moschovakis *et al.*, 1988;Ma *et al.*, 1990).

Tectonigral projection laterality—Case 25 received a small injection of WGA-HRP located in VTA and the adjacent dorsal tier of medial pars compacta (Fig. 9A). Since this was the only example that received a unilateral injection, our retrograde evidence for the laterality of the projection rests on this case. Nevertheless, in agreement with the anterograde data above, an ipsilateral predominance in the tectonigral projection was clear, as 82% of the retrogradely labelled cells were found in the ipsilateral SC. The distribution of the contralateral labelling in this case represented a weak mirror image of that seen ipsilaterally, with an overwhelming majority of cells on both sides (97 and 96% respectively) confined to the intermediate and deep layers. Assuming comparability between medial and lateral injection sites, these findings suggest that a small portion of the labelled cells observed in the bilateral injection cases described below represents crossed projections.

Regional distribution of tectonigral cells—In all cases, the overwhelming proportion of retrogradely labelled tectonigral neurons were confined to the intermediate and deep layers of the SC, as depicted in cases 25 and 91 (Fig. 9A and B, respectively). However, in one case (72L, Fig. 7B), an additional population of labelled neurons was found in the ipsilateral superficial layers. A possible reason for this was that the dorsolaterally placed injection site encroached into the lateral accessory nucleus of the optic tract. In 8/9 cases (Case 25 - Fig. 9A – being the exception) the density of retrograde labelling in rostral sections of the SC was clearly stronger than that observed in the caudal ones (see Case 91 – Fig. 9B as a typical example). Across individual layers in the deep SC, and in the mediolateral dimension, the distribution of retrogradely labelled cells in all 9 cases was relatively even (Fig. 9A&B).

Preferential targeting of the dorsal tier—Given that all 9 injection sites had roughly similar dimensions (Fig. 7B), variability in the total numbers of retrogradely labelled cells detected in different cases, as measured in 3 representative sections, may be attributed to the location of an injection site rather than to its size or shape. The dorsomedial injection in Case 25 was the only example in which the injected tracer was largely confined to dorsal tier DA neurons. It is therefore noteworthy that of all the injections confined to SN, it was associated with the greatest number of labelled ipsilateral tectonigral neurons (a total of 1,687 cells). A second case with a more laterally placed injection site that extended from SNr dorsally into an area containing dorsal tier DA neurons also produced large numbers of retrogradely labelled cells in the SC (837 cells, Case 91R - Fig. 9). In contrast, similarly sized injections directed more ventrally, into areas containing concentrations of ventral tier DA neurons and/or the DA cell columns in SNr, were associated with fewer retrogradely labelled neurons in the ipsilateral SC (Case 91L, 395 cells - Fig. 9; and Case 68R, 516 cells, not illustrated). An injection largely confined to SNr was also associated with retrograde labelling in the SC, albeit at similarly reduced levels (Case 68L, 407 cells not illustrated).

Taken together these results indicate, first, that injections of retrograde tracer entirely confined to substantia nigra were associated with significant levels of retrograde labelling of tectonigral neurons. Secondly, the density of retrograde labelling in the SC was greater for injection sites involving dorsal tier DA neurons, in agreement with the anterograde data. However, the anterograde tracers also revealed that many of the afferent fibres innervating more ventral regions of substantia nigra pass through the dorsal tier. Thus, it is possible that the retrogradely labelled population from more dorsally located injections could include cells whose fibers were labelled on their way to more ventral targets. Thirdly, in all cases, retrogradely labelled neurons were mainly small to medium sized and located in the intermediate and deep layers of the SC. Finally, in a majority of cases, rostral sections of the SC were more heavily labelled.

Discussion

This is the first study to demonstrate a direct efferent projection from the superior colliculus to the substantia nigra in primates. The principal findings were: (i) Anterograde tracer injections into the SC labelled fibers and terminals in SNc, and to a lesser extent in SNr and VTA. (ii) Labelled terminal boutons were observed in close association with TH[+] neuronal elements, particularly within the dorsal tier of DA neurons. (iii) WGA-HRP injections confined to SN retrogradely labelled 4 distinct classes of cells in the intermediate and deep layers of the SC. The relationship to these findings to those of previous investigations and their functional implications will be discussed below.

Cells of origin of the tectonigral pathway

In both cat (McHaffie *et al.*, 2006) and monkey (Fig. 8), the overwhelming majority of tectonigral neurons belong to a few restricted subcategories of the cells present in the SC (Ma *et al.*, 1990); those with small ovoid, bipolar or pyramidal somata, or medium-sized multipolar somata. The small number of larger neurons observed in the cat (McHaffie *et al.*, 2006) were absent in the monkey. This may reflect a genuine species difference, or that nearby tissue targeted by the ipsilateral descending projections was involved by the cat nigral injections (McHaffie *et al.*, 2006). The diversity of labelled neurons in both species suggests the tectonigral projection provides a heterogeneous input to SN, since the different morphological classes are likely to exhibit different patterns of activity in response to stimuli. Such differences could explain why DA neurons might respond differently to various categories of visual event; e.g., appearance, disappearance, movement (Wurtz & Albano, 1980), and looming (Westby *et al.*, 1990).

It is noteworthy that few tectonigral neurons were found in the exclusively visual, superficial layers of the SC (May, 2006). Moreover, the tectonigral population did not include the large tectoreticulospinal output neurons (Moschovakis *et al.*, 1988; May & Porter, 1992). The former finding suggests that tectonigral cells are not exclusively visual, but are instead probably multisensory neurons tuned to the novelty and saliency presets present in the SC (Stein & Meredith, 1993; Stein & Stanford, 2008). The absence of large, retrogradely labelled, output cells is consistent with the general failure to find motor-related activity in DA neurons (Schultz, 1998), including that associated with shifts of gaze (Wurtz & Albano, 1980; Sparks, 1986). Instead, these cells may include the 40% of SGI units that increase the gain of their activity for reward-related responses, but which do not show motor-related activity (Ikeda & Hikosaka, 2003).

Projections to pars reticulata, a possible feedback pathway

The present study provides evidence for a direct tectal projection to SNr. Specifically, terminals in SNr were labelled with either anterograde tracer, and tectal cells were labelled after injections centered in SNr (Case 68L). Due to deliberately weak TH staining, we were unable to determine whether PhaL labelled terminals in pars reticulata made contact with the dendrites of ventral tier SNc neurons. Thus, it is possible that tectal input to SNr may target both DA and GABAergic elements. Supporting this view are the ultrastructural findings in rat that show tectonigral terminals contacting both DA and non-DA profiles (Comoli *et al.*, 2003). While ultrastructural verification is necessary to prove synaptic contacts, the presence of tectonigral boutons in close association with nigrotectal output neurons (Fig. 3C) raises the possibility that the SC modulates the afferent signals it receives from the basal ganglia. It is well established that the nigrotectal projection provides a pathway whereby the basal ganglia influence the gaze-related activity of the SC (Graybiel, 1978; Chevalier *et al.*, 1981; Hikosaka & Wurtz, 1983; Huerta *et al.*, 1991). Furthermore, this pathway is known to target the cells projecting to brainstem gaze centers via the predorsal bundle (May & Hall, 1984; Bickford & Hall, 1992; Jiang *et al.*, 2003). It is therefore possible that tectonigral input to SNr could modulate the disinhibitory output signals from the basal ganglia that gate SC saccade-related outputs (Chevalier & Deniau, 1990; Hikosaka *et al.*, 2000).

Differential projections to dopaminergic neurons

The demonstration of a tectonigral projection in a primate suggests it is a conserved mammalian feature, and therefore likely also to be present in humans. Furthermore, the results of the anterograde and retrograde tracing experiments suggest that the dorsal tier of DA neurons receives a denser tectonigral projection. It is possible that the level of innervation from the SC is related to the relative density of DA neurons in different sectors of substantia nigra. Thus, the greater number of terminals simply reflects a greater number

of targets. Alternatively, it is possible that the dorsal tier of DA is preferentially, although not exclusively, targeted by afferent fibres from the SC. Similar targeting of dorsal tier neurons is a feature of inputs from limbic regions (Lynd-Balta & Haber, 1994; Haber *et al.*, 1995; Fudge & Haber, 2000, 2001). The dorsal tier cells, along with VTA cells, in turn preferentially innervate ventral striatal, and cortical structures (Haber *et al.*, 1995; Haber *et al.*, 2000). Thus, the tectonigral input to these neurons could provide the means whereby biologically salient sensory stimuli might elicit phasic DA modulation in cortico-limbic territories, and through the nigro-striato-nigral system, sequentially influence the dorsal striatum (Belin & Everitt, 2008). This could be of importance for reinforcing cognitive and/ or affective representations associated with the unpredicted occurrence of biologically salient sensory events. This possibility has already been included in a recent model (Gruber *et al.*, 2006), in which it was hypothesized that dopamine acts to gate access to working memory in prefrontal corticostriatal loops.

Pre-attentive sensory input to DA neurons

In all species examined (rat, cat and macaque monkey), it is the SC, rather than cortical systems, that provides the most direct visual input to midbrain DA neurons (Comoli *et al.*, 2003; McHaffie *et al.*, 2006). Consequently, subcortical visual processing is the most likely, if not the exclusive, source of the short latency visual drive for phasic DA signalling (Comoli *et al.*, 2003; Dommett *et al.*, 2005). However, as the brain's 'sentinel', the SC is activated by biologically salient stimuli irrespective of valence, i.e., it is sensitive both to sensory stimuli that elicit defensive, avoidance responses, as well as stimuli that evoke orienting and appetitive approach responses (Dean *et al.*, 1989). Furthermore, direct stimulation of the SC can elicit either defense- or orienting-like movements (Sahibzada *et al.*, 1986; Sparks, 1986; Cole *et al.*, 2006). In contrast, midbrain DA neurons are sensitive at least to a crude form of valence. They respond at pre-attentive latencies to novel or reward-related stimuli with a positive burst of activity (Horvitz *et al.*, 1997; Schultz, 1998) and to noxious (Ungless *et al.*, 2004; Coizet *et al.*, 2006) or detrimental stimuli (Bayer *et al.*, 2007; Matsumoto & Hikosaka, 2007) with a pause or negative response. An important unresolved question is that of how SC representations of potentially appetitive and aversive stimuli could elicit the appropriate positive/negative response in DA neurons. A possible answer could lie in the discovery of multiple tectonigral cell-types in cat (McHaffie *et al.*, 2006) and monkey (present study), which correlates with the fact that tectonigral terminals form both asymmetric (presumed excitatory) and symmetric (presumed inhibitory) synapses onto both TH^+ and TH^- neuronal elements in rat SN (Comoli *et al.*, 2003). Thus, the SC could provide both direct and (via SNr) indirect inputs for excitatory and/or inhibitory influences over ventral midbrain DA neurons. Signals relayed via the tectonigral projection may not, however, provide a complete explanation of the bi-directional phasic responses of DA neurons, as the lateral habenula also appears to be involved in phasic suppression of DA neurons (Matsumoto & Hikosaka, 2007).

Role of tectonigral input in reinforcement learning

Here we have demonstrated a tectonigral pathway which appears to terminate in a distributed fashion upon TH^+ neurons in SNc. It is likely that this pathway is responsible for the short latency (<100 ms), phasic visual signals expressed by DA neurons, as visual activity is present in the intermediate layer of the superior colliculus at latencies of less than 100 ms (Wurtz & Albano, 1980; Jay & Sparks, 1987). Other prospective sources of visual input where the identity of a visual stimuli are ascertained, such as the P-pathways through the geniculostriate visual system to infra-temporal cortex, could provide such information, but these sources have latencies for activity that are too long to explain the short latency DA activity (Thorpe & Fabre-Thorpe, 2001). This situation would be compounded by the fact foveating eye movements are often necessary for identification of targets. Furthermore,

direct contacts between infra-temporal cortex and the ventral midbrain are absent, so appropriate connections would require further relays through pre-frontal cortex (Sesack & Carr, 2002; Frankle *et al.*, 2006) and/or the striatum (Middleton & Strick, 1996). Finally, it has been shown that visual cortex is not necessary for short latency visual activity (Dommett *et al.*, 2005). Instead, the visual response in DA neurons is due to retinal input to SGS, as the effects of the tectonigral projection are dependent upon activity within this layer of the SC (Comoli *et al.*, 2003). Presumably, this visual information is provided to tectonigral neurons in SGI via intralaminar pathways (Hall & Lee, 1997; Isa, 2002).

In this light, the characteristics of the tectonigral visual inputs are particularly relevant. While the physiology of this projection has not been specifically investigated, we do have a general understanding of collicular visual sensory processing (Wurtz & Albano, 1980; Sparks, 1986; Stein & Meredith, 1993; Stein & Stanford, 2008). Within the SC, visual sensory neurons tend to be insensitive to static features, color, and high spatial frequencies, but are exquisitely sensitive to the appearance/disappearance of stimuli and their movement (Wurtz & Albano, 1980). These visual responses often quickly habituate, but there is an enhancement of this activity when the stimulus is used as a target for a rewarded behaviour (Goldberg & Wurtz, 1972). In addition, these responses may be relayed more effectively from superficial to the deep layers of the colliculus when the stimulus is a rewarded target (Isa, 2002). The location of stimuli is encoded by the position of the activated population of collicular neurons within the topographical, retinotopic representation of space. However, this characteristic is probably not transmitted to SNc, as the tectonigral projection appears to be quite diffuse and non-topographic. Thus, if this signal contributes to reinforcement learning mechanisms within the SNc, it would seem to primarily convey information about the occurrence of an event, with relatively little information about its identity or location.

The present anatomical study cannot, in itself, prove or disprove any particular hypothesis of DA signalling, since there are many other sources of non-visual input to DA cells (Kitai *et al.*, 1999; Grace *et al.*, 2007). Nevertheless, the possibility that pre-attentive visual activation of DA neurons occurs via the tectonigral projection raises important questions concerning the role of these cells in reinforcement learning. For example, unlike many of the paradigms commonly used to demonstrate a tight relationship between dopamine signaling and mathematically defined reward prediction errors (Schultz, 2002; Fiorillo *et al.*, 2003; Tobler *et al.*, 2003; Tobler *et al.*, 2005), in real world circumstances, sensory events can be unpredictable in kind, space and time. Under such circumstances, and provided the colliculus is the source of visual input, it is unlikely that the identity (and hence the economic value) of stimuli are known at the time the phasic DA signal (Redgrave & Gurney, 2006). Rather, stimulus identity and economic utility are more likely to be determined by sophisticated cortical perceptual systems after a foveating gaze shift (Thorpe & Fabre-Thorpe, 2001). It is probably safer, therefore, to consider phasic DA signals provided by the colliculus as 'sensory prediction errors', rather than 'reward prediction errors', as is currently posited (Montague *et al.*, 2004; Schultz, 2006; D'Ardenne *et al.*, 2008). We have argued (Redgrave *et al.*, 2007) that crude valence information available to DA neurons at short latency in the form of sensory prediction errors would be appropriate to reinforce: (i) the discovery of agency; and (ii) the development of novel actions. In the former, the phasic tectonigral input to DA neurons would be used to reinforce the discrimination between externally- and self-generated events. In the latter, the phasic DA response may be used in the process that can identify the specific components in the behavioural output which caused the unpredicted outcome. In this light, instrumental conditioning may resolve into two processes that rely on different mechanisms of reinforcement: (i) discovery of agency and the development of novel actions, which are reinforced by phasic dopamine signals from the superior colliculus (Redgrave & Gurney, 2006); and (ii) reward-based Law of Effect learning, which adjusts the probability of

selecting future actions to maximise economic value (Thorndike, 1911). The reinforcement signals for Law of Effect learning are currently unknown, but a good place to start looking would be in perceptual systems that perform detailed discriminations of economic value after foveation (Furuyashiki & Gallagher, 2007; Lee & Seo, 2007; Salzman *et al.*, 2007; Padoa-Schioppa & Assad, 2008).

Acknowledgments

Abbreviations

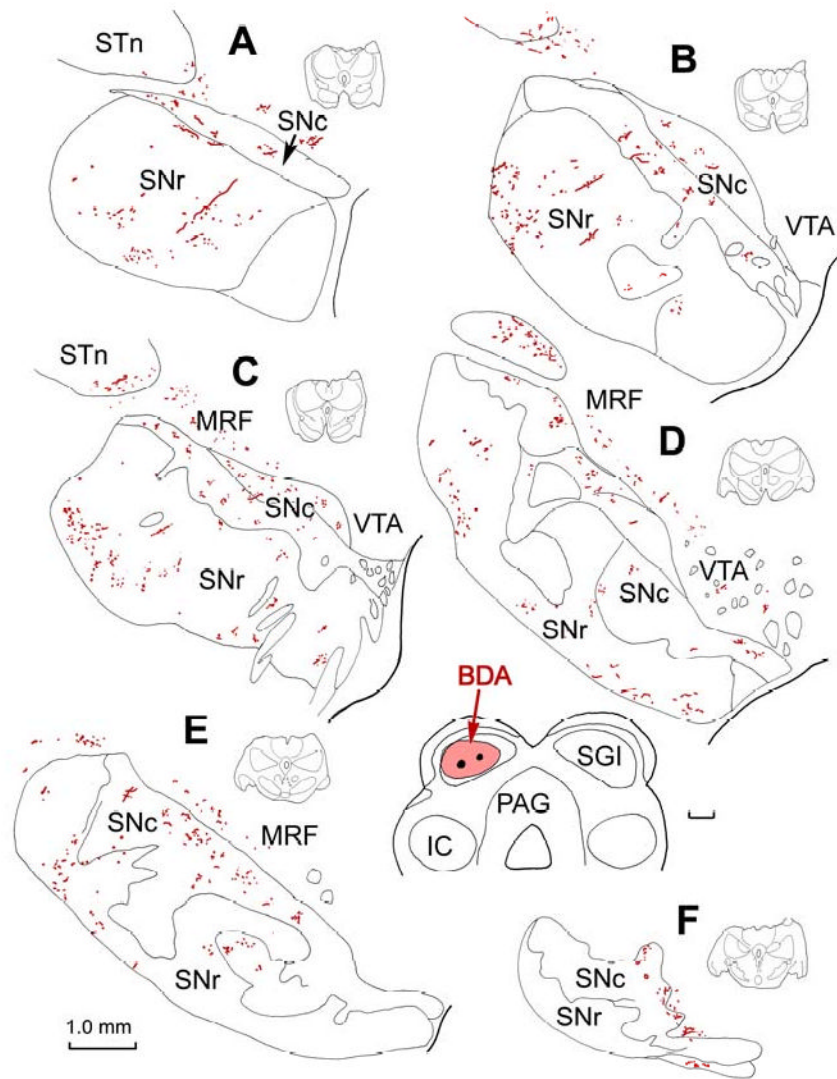|	
BC	brachium conjunctivum
BDA	biotinylated dextran amine
DA	dopaminergic
DAB	diaminobenzidine HCl
HRP	horseradish peroxidase
IC	inferior colliculus
IIIn	third cranial nerve
MRF	midbrain reticular formation
NGS	normal goat serum
PAG	periaqueductal gray
PB	phosphate buffer
PBS	phosphate buffered saline
PBS-T	PBS plus Triton-X 100
PhaL	*Phaseolus vulgaris* leucoagglutinin
SC	superior colliculus
SAI	intermediate white layer
SGI	intermediate gray layer
SGP	deep gray layer
SGS	superficial gray layer
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SO	stratum opticum
STn	subthlamic nucleus
TH	tyrosine hydroxylase
VTA	ventral tegmental area
WGA-HRP	wheat germ agglutinin conjugated HRP

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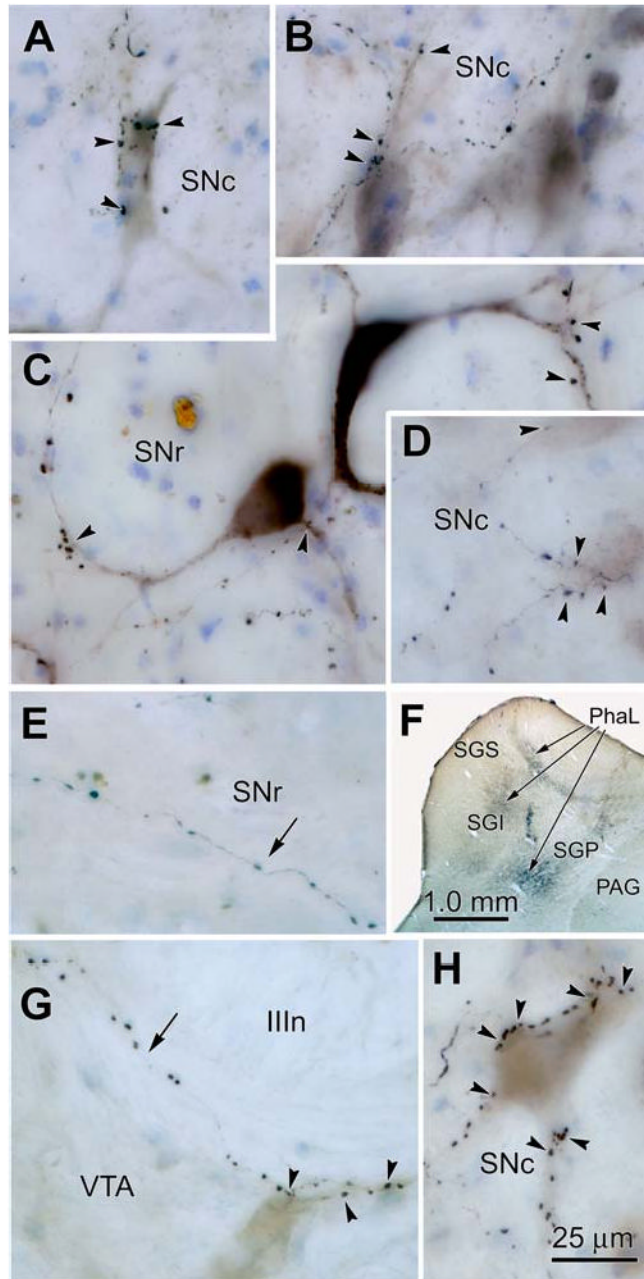
Figure 1.
Distribution of labelled tectonigral axons in the substantia nigra. An injection of BDA centered in SGI of the SC (insert, lower right) anterogradely labelled axons throughout the SN, as shown in a rostral to caudal series of frontal sections (A-F). The illustrated levels are indicated by the small schematics to the right of each illustration. Labelled axons are present in both SNc and SNr, as well as in the adjacent midbrain reticular formation, ventral tegmental area, and subthalamic nucleus. Abbreviations used in this in subsequent figures: BC - brachium conjunctivum; BDA - biotinylated dextran amine; IC - inferior colliculus; IIIn - third cranial nerve; MRF - midbrain reticular formation; PAG - periaqueductal gray; PhaL - *phaseolus vulgaris* leucoagglutinin; SC - superior colliculus; SGI - intermediate gray layer; SGP - deep layer; SGS - superficial gray layer; SN - substantia nigra; SNc - substantia nigra pars compacta; SNr - substantia nigra pars reticulata; SO – stratum opticum; STn - subthlamic nucleus; VTA - ventral tegmental area.
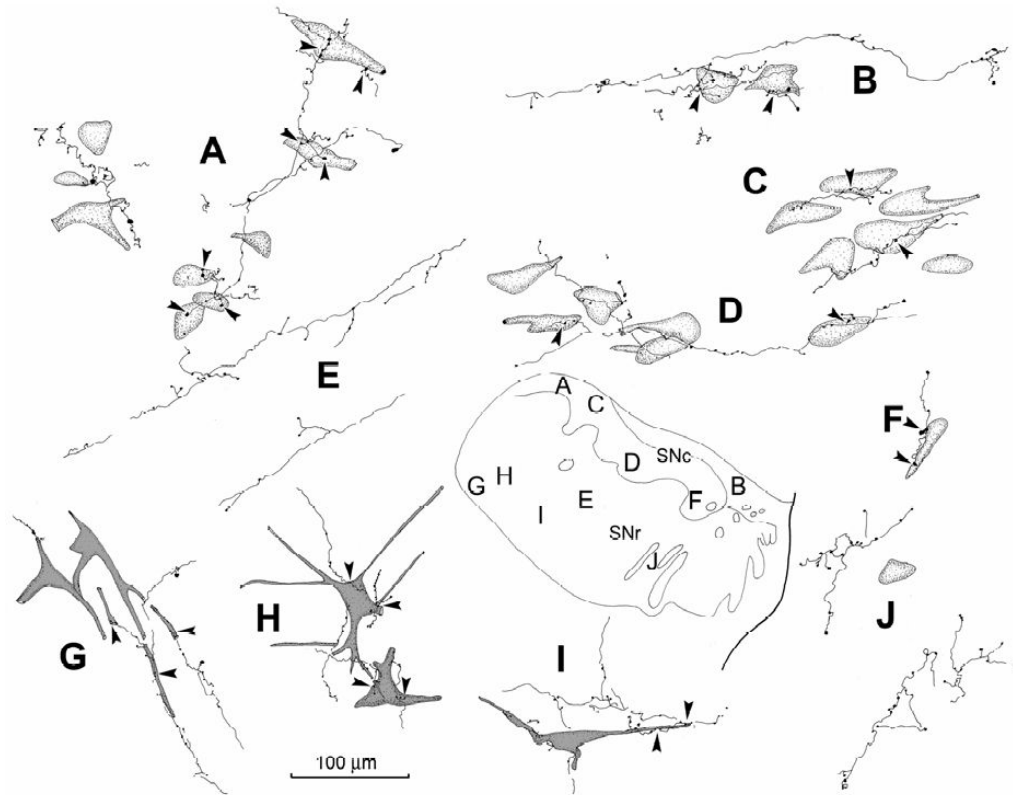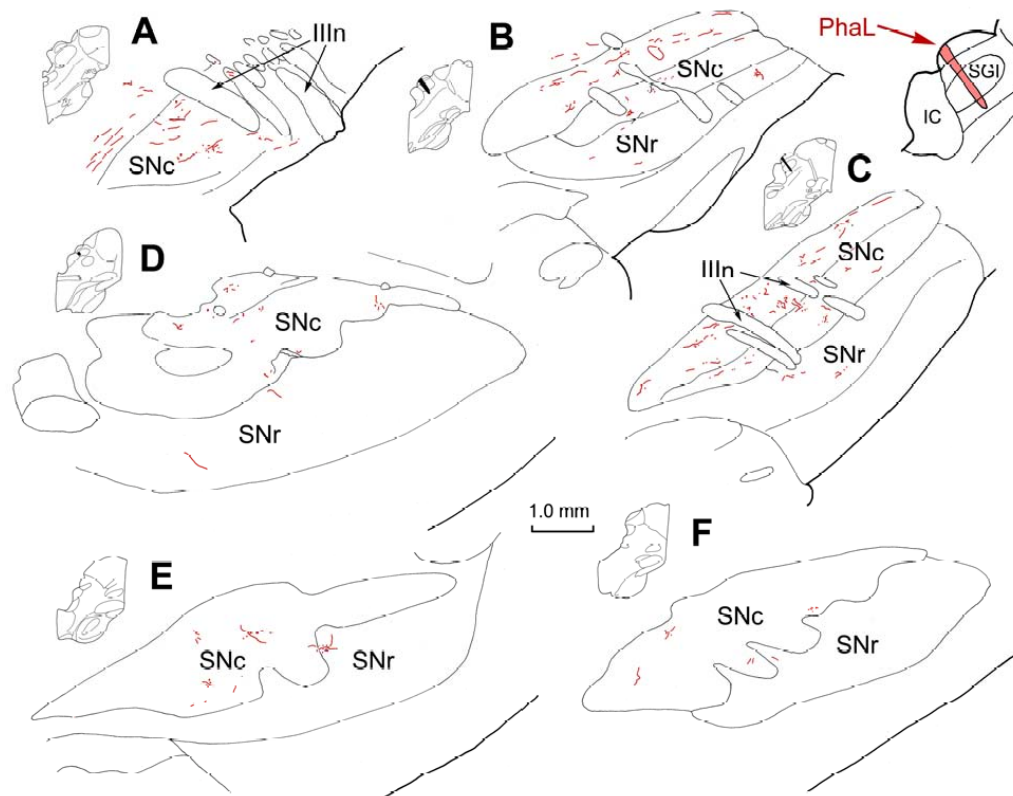
Figure 2.
Relationship of BDA labeled tectonigral terminal arbors to components of SN. Individual arbors labelled from the injection illustrated in figure 1 are shown. BDA labelled tectonigral axons were distributed among counter-stained somata in SNc (A-D&F). They were also found in SNr (E&G-J), where they were sometimes associated with retrogradely labelled nigrotectal neurons (G-I). These axons displayed boutonal enlargements that sometimes lay in close association (arrowheads) with cells in SNc and SNr. The location of each illustrated example is shown in the schematic (lower right).

Figure 3.
Relationship between BDA (A-C) and PhaL (D,E,G&H) labelled tectonigral boutons and nigral (A-D&H) and VTA (G) neurons. The BDA injection shown in figure 1 produced the labelled tectonigral axons shown in A-C. The boutons of these axons displayed close associations (arrowheads) with ipsilateral SNc neurons (A&B) which contained brown neuromelanin granules. In SNr, anterogradely labelled tectonigral boutons displayed numerous close associations (C) with retrogradely labelled nigrotectal cells, in which dark brown reaction product extended into the dendrites. The labelled axons in E, D, G & H were produced by the PhaL injections shown in F. D is from the contralateral SNc. E shows a PhaL labelled axon in SNr (arrow) that lay parallel to and just above the cerebral peduncle.

A labelled axon in the VTA (G, arrow) skirts a fascicle of the oculomotor nerve and shows close associations with a neuron that has numerous neuromelanin granules. The two SNc neurons shown in H are unusual, in that they had numerous labelled boutons in close association with them. Number of z-axis planes: A=11; B=20; C=20; D=10; E=6; F=1; G=7; H=9.
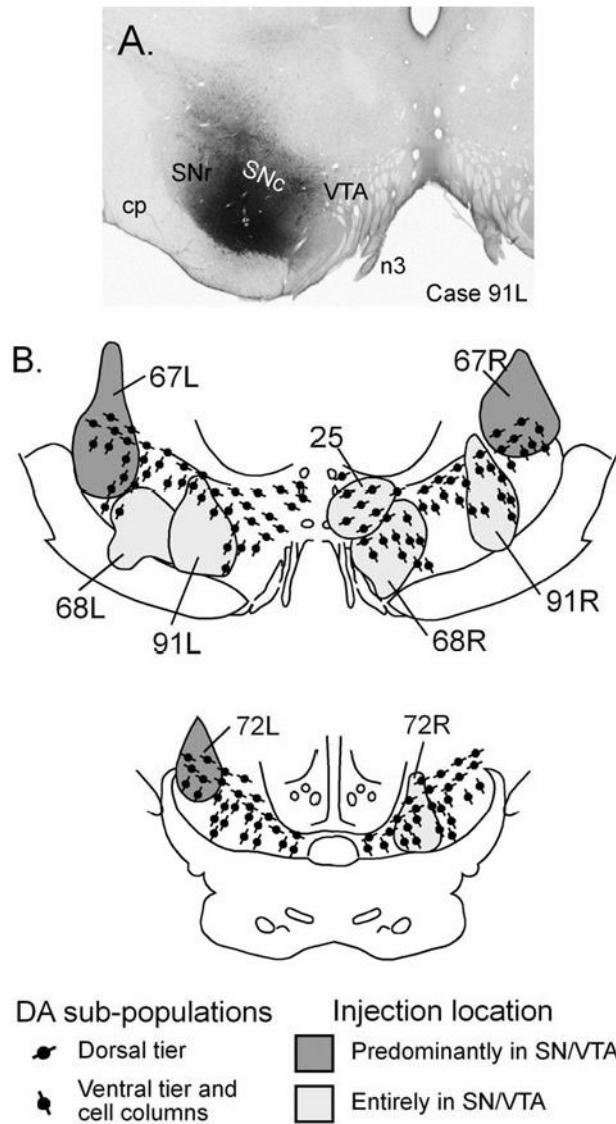
Figure 4.
Distribution of labelled tectonigral axons in SN shown in the parasagittal plane. The injection of PhaL, which involved all layers of SC (insert, upper right), anterogradely labelled axons throughout the SN, as shown in a medial to lateral series of parasagittal sections (A-F). The illustrated levels are indicated by the small schematics to the left of each illustration. Labelled axons were present in both SNr and SNc, and were densest in the dorsal tier of SNc.
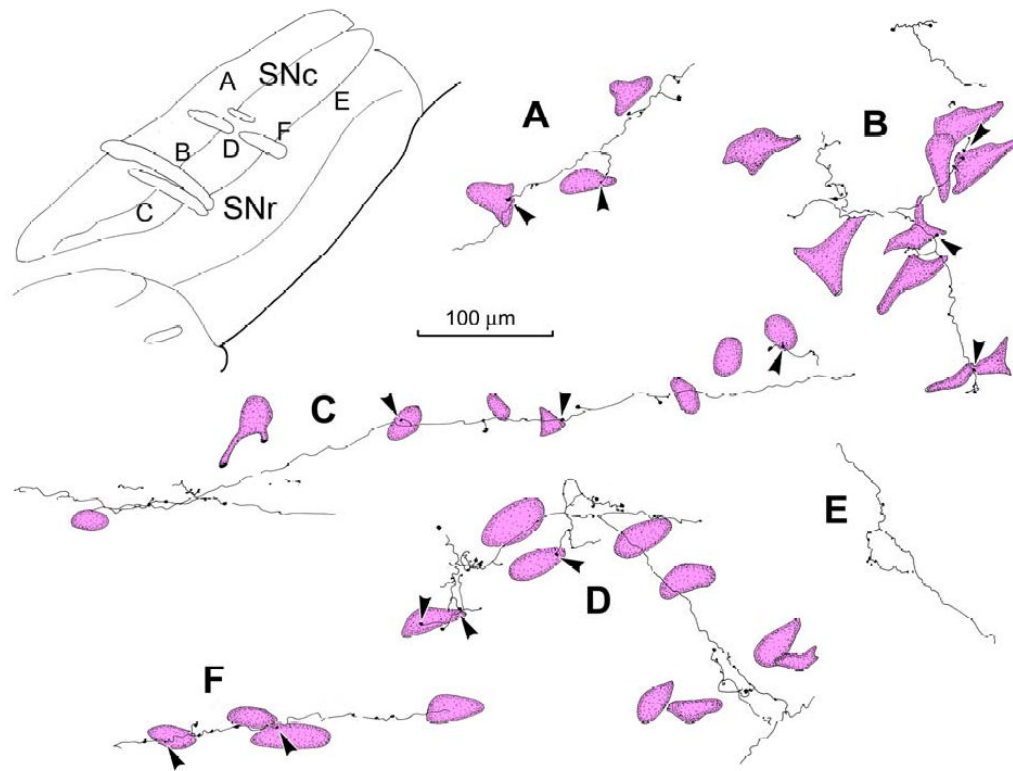
Figure 5.
Morphology of PhaL labeled tectonigral terminal arbors and their relationship to TH[+], presumed DA, neurons. Individual arbors labeled from the injection illustrated in figure 4 are shown. PhaL labelled tectonigral arbors were distributed among TH^{+} neurons in SNc (A-D&F), and displayed boutons with close associations (arrowheads) to some cells. They were also found in pars reticulata (E). The location of each illustrated example is shown in the schematic of a parasagittal section (upper right).
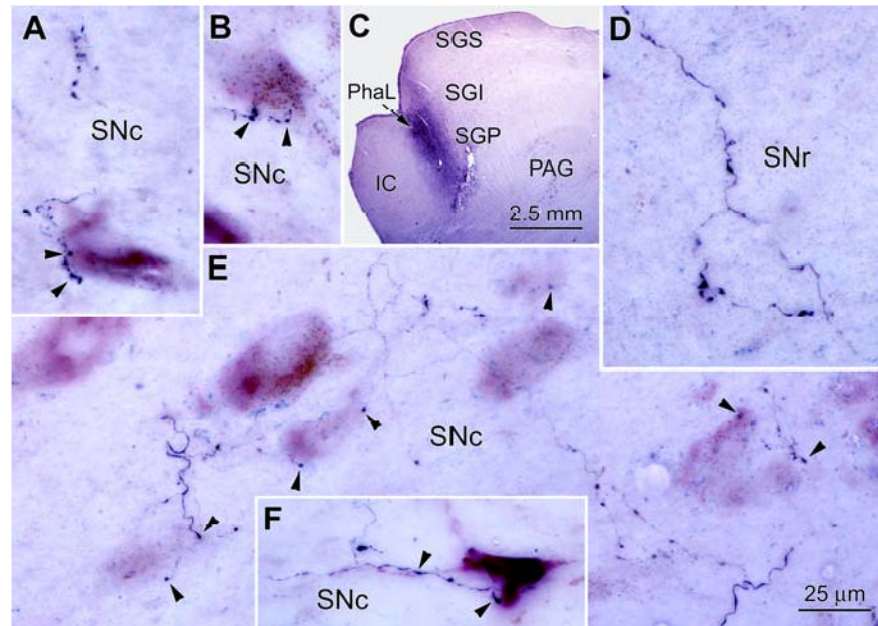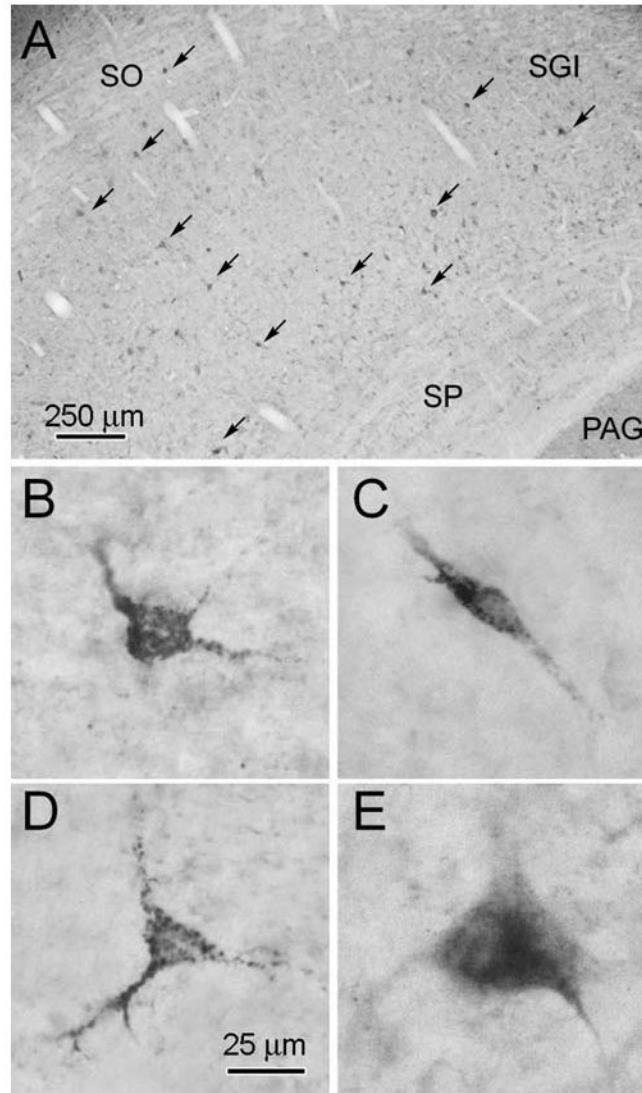
Figure 6.
Phal labelled terminal arbors in the SN. PhaL labeled tectonigral axons (black) from the injections shown in Fig. 4 and in C are illustrated in D&E and in A,B&F, respectively. They displayed numerous boutons in SNc (A,B,E&F) and SNr (D). The formert had close associations (arrowheads) with VIP stained (purple) TH$^+$ cells in SNc (A,B,E&F). The morphology of the TH$^+$ cells is accentuated by the presence of brown neuromelanin granules in the cytoplasm. Number of z-axis planes: A=3; B=10; C=1; D=9; E=8; F=11.
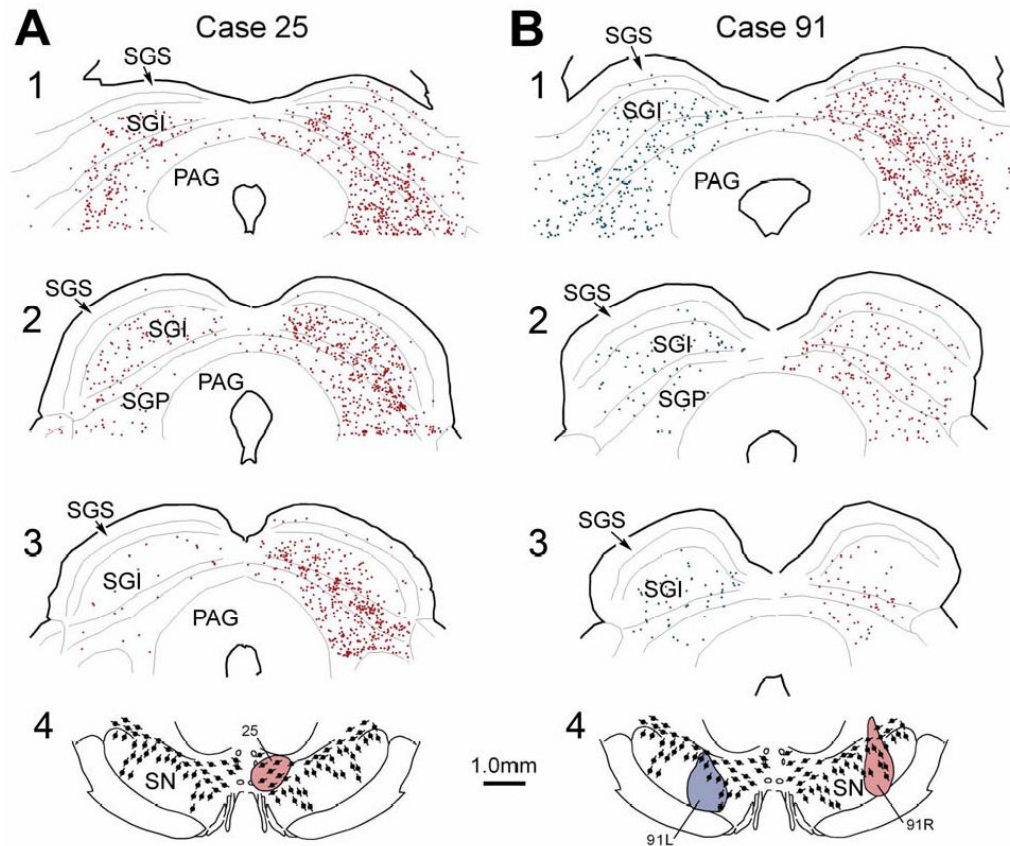
Figure 7.
WGA-HRP injection sites in the SN and VTA. An example of an injection confined to the SN is shown in A. B&C are rostral and caudal frontal sections displaying the widest extent of each of the injection sites used in this study. The light gray injection sites were confined to the SN and VTA, while dark gray injection sites extended into the overlying midbrain reticular formation.

Figure 8.
Distribution and morphology of retrogradely labelled tectonigral neurons. The labelled neurons were mainly found in the layers beneath stratum opticum (A). They fell into four morphological classes: small ovoid (B), small fusiform (C), small pyramidal (D) and medium-sized multipolar (E).

Figure 9.
The distribution of retrogradely labelled tectonigral neurons in the SC. A illustrates the location of ipsilateral and contralateral labelled neurons in a rostrocaudal series of frontal sections (A1-3) through the SC following a WGA-HRP injection confined to the VTA and dorsal tier of the SNc (A4). B illustrates the distribution pattern (B1-3) in an animal that received bilateral injections of SN (B4). Cells from 3 adjacent sections were plotted onto each level shown.