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Inputs to the Midbrain Dopaminergic Complex in the Rat with Emphasis on Extended Amygdala-recipient Sectors

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Abstract

The midbrain dopaminergic neuronal groups A8, A9, A10 and A10dc occupy, respectively, the retrorubral field (RRF), substantia nigra compacta (SNc), ventral tegmental area (VTA) and ventrolateral periaqueductal gray (PAGvl). Collectively, these structures give rise to a mixed dopaminergic and non-dopaminergic projection system that essentially permits adaptive behavior. Yet, knowledge is incomplete regarding how the afferents of these structures are organized. While the VTA is known to get numerous afferents from cortex, basal forebrain and brainstem and the

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SNC is widely perceived as receiving inputs mainly from the striatum, the afferents of the RRF and PAGvl have yet to be addressed comprehensively. This study was done to provide an account of those connections and seek a better understanding of how afferents might contribute to the functional interrelatedness of the VTA, SNC, RRF and PAGvl. Ventral midbrain structures received injections of retrograde tracer and resulting retrogradely labeled structures were targeted with injections of anterogradely transported *Phaseolus vulgaris*-leucoagglutinin. While all injections of retrograde tracer into the VTA, SNC, RRF or PAGvl produced labeling in many of a long list of structures extending from the cortex to caudal brainstem, pronounced labeling of structures comprising the central division of the extended amygdala occurred following injections that involved the RRF and PAGvl. The anterograde tracing supported this finding and, interestingly, the combination of retrograde and anterograde labeling data also confirmed reports from other groups indicating that the SNC receives robust input from many of the same structures that innervate the VTA, RRF and PAGvl.

Keywords

retrobulbar field; ventral tegmental area; substantia nigra compacta; periaqueductal gray; amygdala; bed nucleus of stria terminalis

Catecholamine-containing neurons and axon pathways in the rat brain were mapped with the aid of aldehyde-induced fluorescence in the mid-nineteen sixties and since have ranked continuously among the most studied of all brain structures. Dopaminergic neuronal groups A8, A9, A10 (Dahlström and Fuxe, 1964; Anden et al., 1964, 1965; 1966a; 1966b; Björklund et al., 1984) and A10dc (Hökfelt et al., 1984) occupy, respectively, the retrorubral field (RRF), substantia nigra compacta (SNC), ventral tegmental area (VTA) and ventral and lateral parts of the periaqueductal gray (PAGvl). While these structures are invariably referred to as “dopaminergic”, all are supplemented by indeterminate proportions of intermixed dopaminergic neurons co-expressing glutamate (Lapish et al., 2006, Hnasko et al., 2010; Tecuapetla et al., 2010) and non-dopaminergic neurons (Swanson, 1982) expressing gamma-aminobutyric acid (GABA, Steffensen et al., 1998) or glutamate (Yamaguchi et al., 2007; Dobi et al., 2010) that may give rise to long projections and interact with dopaminergic neurons locally. The output from these structures, which we will herein continue to refer to as the midbrain dopaminergic complex, contributes to a broad range of functions, including but not limited to locomotor activation (e.g., Kelly et al., 1975), stimulus and response reinforcement (e.g., Wise, 2004) and fear conditioning (Guarraci et al., 1999, 2000; Fadok et al., 2009) and thus is essential to the neural processes that subserve adaptive behavior.

Whereas the SNC projects mainly to the dorsal striatum, the VTA innervates the ventral striatum, septum, basal forebrain, basal amygdala, ventral hippocampus and medial prefrontal cortex (Fallon et al., 1978; Fallon and Moore, 1978; Beckstead et al., 1979; Swanson, 1982; Lindvall and Björklund, 1983; Phillipson and Griffiths, 1985; Fallon and Loughlin, 1985; 1995; Fallon, 1988; Del Fava et al., 2007) and the RRF projects to dorsal and ventral striatum as well as the piriform and entorhinal cortices (Deutch et al., 1988). The VTA and RRF also project to the extended amygdala (Deutch et al., 1988; Hasue and Shammah-Lagnado, 2002), which includes the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BST) and some related structures (de Olmos and Ingram, 1972; Alheid and Heimer, 1988; de Olmos and Heimer, 1999). However, the PAGvl, which is reported to contain neurons that utilize L-DOPA in place of dopamine (Hökfelt et al., 1984; Misu et al., 1996), appears to have the most catecholaminergic neurons projecting to extended amygdala (Hasue and Shammah-Lagnado, 2002).

As regards afferents, the VTA is most studied among the components of the midbrain dopaminergic complex. VTA inputs arise in multiple structures in the cortex, basal forebrain and brainstem (Nauta and Domesick, 1978; Phillipson, 1979; Wallace et al., 1989; 1992; Carr and Sesack, 1999; 2000; Zahm et al., 2001; Fadel and Deutch, 2002; Philpot et al., 2005; Olmechenko and Sesack, 2005; 2006; 2007; 2009; 2010; Balcita-Pedicino and Sesack, 2007; Ferreira et al., 2008; Olmechenko et al., 2009) reported to comprise a highly interconnected network (Geisler and Zahm, 2005; 2006; Geisler et al., 2007). The SNc, in contrast, is widely regarded as getting input mainly from the dorsal striatum (Beckstead et al., 1979; Gerfen et al., 1985; Jimenez-Castellanos and Graybiel, 1989), despite numerous reports of a lateralward spread through the SNc of a variety of afferents that occupy the medial forebrain bundle (Nauta and Domesick, 1978; Nauta et al., 1978; Krettek and Price, 1978; Nauta and Domesick, 1984; van der Kooy et al., 1984; Zahm and Heimer, 1990; 1993; Gonzales and Chesselet, 1990; Heimer et al., 1991b; Rosen et al., 1991; Zahm et al., 1999; Dong et al., 2001; Gastard et al., 2002; Coizet et al., 2010; Geerling et al., 2010). Behind the SNc, the medial forebrain bundle arches through the lateral RRF and then turns caudalward to pass along and emit fibers into the PAGvl. The afferents of neither the RRF nor PAGvl have been comprehensively described in the literature. The experiments described herein and accompanying literature review thus were done primarily to identify the afferents of the RRF and PAGvl and secondarily to develop a better picture of how the midbrain dopaminergic complex, considered as a whole, is innervated.

Materials and Methods

Male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 225–300 g were used in accordance with guidelines mandated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The rats were housed on a 12 hr light-dark cycle in groups of four until surgeries were performed, after which they were singly housed. Access to food and water was provided ad libitum to all rats throughout the study. Unless stated otherwise, chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Tracer injections

Several minutes after being given intraperitoneal injections of a cocktail, consisting of 45% ketamine (100 mg/ml), 35% xylazine (20 mg/ml) and 20% physiological saline at a dose of 0.16 ml/100g of body weight, rats were placed in a Kopf stereotaxic instrument. The skulls were exposed and small bore holes were created to allow selected brain structures to be targeted by filament-containing borosilicate glass pipettes (O.D. – 1.0 mm) pulled to tip diameters of 10–25 μ m and containing the retrograde tracer Fluorogold (FG; Fluorochrome, Inc., Englewood, CO; 1% in 0.1M cacodylate buffer, pH 7.4) or an anterograde tracer, either *Phaseolus vulgaris*-leucoagglutinin (PHA-L; Vector, Burlingame, CA, 2.5% in 0.01 M phosphate buffer) or biotinylated dextran amine (BDA, Molecular Probes, Inc., Eugene, OR, 10% in 0.01 M phosphate buffer). A silver wire inserted into the pipettes contacted the solution containing the tracer, which was ejected into the brain substance using positive current pulses (7 s on, 7 s off, for 15 minutes) of 1 μ A (for FG) or 4 μ A (for PHA-L and BDA). After surgery the rats were kept warm until they awakened.

The analysis utilized 16 injections of FG (e.g., Figs. 1 and 2) evaluated in relation to the midbrain dopaminergic complex, which was revealed with the aid of tyrosine hydroxylase immunoreactivity (Fig. 1). Two of the injections were centered in the PAGvl and one, three and six injections occupied the medial, middle and lateral RRF, respectively. In addition, two injections in the lateral half of the SNc were used, as were two, prepared for an earlier study (Geisler and Zahm, 2005), that occupied about the center of the VTA. A further two control injections into the SNr (05105, 05106) and many into various parts of the midbrain tegmentum near the dopaminergic complex were also studied (06093; 06162, 07120 – red

nucleus and adjacent tegmentum; 06014 – tegmentum lateral to red nucleus and above RRF; 06167 – tegmentum below caudal RRF and ventromedial to the pedunculo-pontine tegmental nucleus; 07086; 07114; 07115, 07130, 08019, 08020, 08023 – rostromedial tegmental nucleus; 07124, 08026 – paramedian raphe; 08022 – midline between rostromedial tegmental nuclei; 07118 – interpeduncular nucleus; 07119 – medial lemniscus; 07126 – oculomotor nucleus). In addition, 31 cases were studied in which PHA-L or BDA injection sites were centered in various forebrain and brainstem structures selected on the basis of the retrograde labeling, such as, e.g., the prefrontal cortex (PHA-L, 04265 and 04266), accumbens (PHA-L, 98003, 98005), ventral pallidum (BDA, 94031, 94032, 94040, 94051, and 94052), subnucleus extended amygdala (PHA-L, 95109), lateral preoptic area (PHA-L, 03153, 06148, 06149, 09108, 09025), lateral hypothalamus (PHA-L, 96049, 96108, 96199), lateral habenula (PHA-L, 07142, 08002, 08004, 08011), zona incerta (PHA-L, 95108), entopeduncular nucleus (PHA-L, 96134) ventral tegmental area (PHA-L, 07046 and 07077) and substantia nigra compacta (PHA-L, 07004 and 07014) and rostromedial tegmental nucleus (PHA-L, 06076, 06096, 07033). Targeting of FG and PHA-L injections was done with the aid of stereotaxic coordinates which were initially acquired from the atlas of Paxinos and Watson (2007) and refined empirically. Most abbreviations used in the text, figures and tables are from Paxinos and Watson (2007).

Fixation of brains and immunocytochemistry

Three days after FG injections, ten days after PHA-L injections and five days after BDA injections, the rats were deeply anesthetized (as above) and perfused transaortically, first with 0.01 M Sorensen's phosphate buffer (SPB; pH 7.4) containing 0.9% sodium chloride and 2.5% sucrose, followed by 0.1 M SPB (pH 7.4) containing 4% paraformaldehyde and 2.5% sucrose. The brains were removed, post-fixed, infiltrated with 25% sucrose and sectioned frozen at 50 μ m. Five adjacent series of sections were collected, with each thus reflecting the structure of the entire brain from frontal pole to caudal medulla in sections spaced at intervals of 250 μ m. Each series of sections was stored in a separate glass vial at -20° C in a cryoprotectant consisting of SPB containing 30% sucrose (by weight) and 30% ethylene glycol (by volume).

One series of sections from each case was immersed in SPB containing 0.1% Triton X-100 (SPB-t) and polyclonal antibodies raised against the relevant tracer, i.e., either anti-FG made in rabbit and used at a dilution of 1:5000 or anti-PHA-L made in goat used at a dilution of 1:10,000. The following day, after thorough rinsing in SPB-t, the sections were immersed for an hour in SPB-t containing biotinylated antibodies made in donkey against rabbit (for FG) or goat (for PHA-L) at a dilution of 1:200 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Afterward, the sections were rinsed in SPB and then immersed in SPB containing avidin-biotin-peroxidase complex (ABC - Vector Laboratories, Burlingame, CA) at a dilution of 1:200, also for an hour. Cases with BDA injections were pretreated by immersion in 1% aqueous sodium borohydride for 15 minutes followed by thorough rinsing and then placed immediately in SPB-t containing ABC reagents at a dilution of 1:200. After additional thorough rinsing in SPB, the sections were immersed for 20–30 min in 0.05 M SPB (pH 7.4) containing 0.05% DAB, 0.04% ammonium chloride, 0.2% β -D-glucose, and 0.0004% glucose oxidase, which generates an insoluble brown reaction product, or, if the sections were destined to be reacted with a second primary antibody, in 0.025 M Tris buffer (pH 8.0) containing 0.015% 3,3'-diaminobenzidine (DAB), 0.4% nickel ammonium sulfate and 0.003% hydrogen peroxide, which generates an insoluble black reaction product.

Sections intended for additional immunocytochemical processing were then further rinsed in SPB and immersed in SPB-t containing, anti-nitric oxide synthase (NOS) or anti-neurotensin (NT) made in rabbit or anti-tyrosine hydroxylase (TH) made in mouse, all used at a dilution of 1:5000. The following morning the sections were rinsed in SPB-t and immersed for one

hour in SPB-t containing a donkey antibody against mouse or rabbit IgGs, as appropriate, each used at a dilution of 1:200 (Jackson). Following further rinsing in SPB the sections were immersed for one hour in SPB containing, respectively, mouse or rabbit peroxidase-anti-peroxidase (PAP) at a dilution of 1:3000 (MP Biomedicals, Solon, OH), after which they were again rinsed thoroughly. Then the sections were immersed for 20–30 min in 0.05 M SPB (pH 7.4) containing 0.05% DAB, 0.04% ammonium chloride, 0.2% β -D-glucose, and 0.0004% glucose oxidase (brown reaction product) or 0.025 M Tris buffer (pH 8.0) containing 0.015% 3,3'-diaminobenzidine (DAB) and 0.003% hydrogen peroxide (also brown) and, after rinsing, mounted onto gelatin coated slides, dehydrated through a series of ascending concentrations of ethanol, transferred into xylene, and coverslipped with Permount (Fisher, Pittsburgh, PA).

Antibody Characterization

The primary antibodies used in the study and their sources are listed in Table 1. Information on the characterization of the antibodies and specificity controls is given below.

anti-FG—Immunoprocessed sections from brains lacking FG injections were devoid of reaction product and immunoreactivity was abolished by preabsorption with FG (10 μ g/ml). In brains that received injections of FG, immunostaining was observed only at the injection sites, in retrogradely labeled neurons and, occasionally, in microglial cells, which, however, are readily distinguished by morphology from labeled neurons. The vendor states that AB153 also reacts with amino-stilbamidine in frozen, 4% PFA fixed tissues.

anti-NT—NT immunoreactivity was abolished in our hands by preabsorption with the cognate peptide (10 μ g/ml). Furthermore, the antibody stained rat forebrain and ventral midbrain sections identically to descriptions in literature (e.g., Uhl et al., 1977).

anti-Nos—Nos immunoreactivity was abolished in our hands by preabsorption with the cognate peptide (10 μ g/ml). Furthermore, the antibody stained rat forebrain and ventral midbrain sections identically to descriptions in literature (e.g., Rodrigo et al., 1994).

anti-PHA-L—Immunoprocessed sections from brains lacking PHA-L injections were devoid of PHA-L reaction product and immunoreactivity was abolished by preabsorption with PHA-L (10 μ g/ml). In brains that received injections of PHA-L, immunostaining was observed only at the injection sites and in anterogradely labeled axons.

anti-TH—In the vendor's Western blots, 1:1000 dilution of the lot used in the study (LV1377069) detected tyrosine hydroxylase on 10 μ g of mouse and rat brain lysates. The antibody stained a single band consistent with a protein of approximately 59–61 kDa. It did not react with the following on Western blots: dopamine-beta-hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase, dehydropteridine reductase, sepiapterin reductase or phenethanolamine-N-methyl transferase (PNMT). In our hands, the antibody stained rat forebrain and ventral midbrain sections identically to descriptions in literature (e.g., Lindvall et al., 1983; Hökfelt et al., 1984).

Maps and photomicrographs

Retrograde and anterograde labeling was plotted in representative frontal sections throughout the CNS (excluding spinal cord) with the 10X or 20X objective under brightfield optics (Nikon Optiphot) with the aid of the AccuStage digitizing system and MDPlot software (AccuStage™, Shoreview, MN) or the Neurolucida dedicated hardware-software platform (MBF Bioscience, Williston, VT). Maps of retrograde labeling included 4 cases representing the medial (case 08024), middle (case 06166) and lateral (case 10197) RRF and

PAGvl (case 10099) of which 31, 27, 27, and 26 levels were plotted, respectively. Sixteen levels of case 06166 (16 levels) are shown in the paper (Fig. 3) as are parts (5 levels) of cases 10197 and 10099 (Fig. 4). Anterograde tracing was mapped using the Neurolucida system in a manner such that only axonal varicosities, i.e., puncta (Figure 5A') were plotted to the complete exclusion of non-varicose, presumably mainly non-synaptic, parts of labeled axons. Varicosities were recognized as distinct, punctate swellings or dilatations of PHA-L filled axons that often were more intensely immunoreactive than adjacent non-varicose parts of labeled axons (see arrowed structures in Fig. 5A'). For anterograde tracing, mapping was limited to the midbrain dopaminergic complex. The following cases and levels were mapped: for BSTl-d - 00022 (6 levels), 99083 (5 levels); for BSTl-i - 99052 (6 levels), 06142 (6 levels), for BSTl-v - 06042 (6 levels), 06043 (4 levels); 06060 (6 levels); for CeAm-m - 00061 (4 levels); for CeAm-l - 11105 (4 levels); for LPO - 03153 (4 levels); 09017 (4 levels), 09018 (4 levels), 09025 (5 levels); for VP - 94052 (6 levels).

Digital micrographs were captured with a Q Imaging Fast 1394 digital camera and adjusted mainly for brightness and contrast with Adobe Photoshop (CS2) software. The plates were constructed using Adobe Illustrator (CS2) software.

Quantitation of anterogradely labeled axonal varicosities

Having been mapped with the aid of the Neurolucida system, varicosities residing in the VTA, SNc, RRF, and PAGvl, which were identified with the aid of tyrosine hydroxylase immunoreactivity in the same or an adjacent section, were circumscribed and counted with the aid of NeuroExplorer software (MBF Bioscience) and expressed as varicosities/mm² within the circumscribed area. This provides density of axonal varicosities specifically within the area of distribution of the projection, rather than relative to the area of the entire structure, an appropriate measurement because topographically distributed projections often do not fill an entire structure. Each evaluated projection was so quantitated in at least 3 levels of the midbrain dopaminergic complex and the measurements were averaged. The averages were used to provide means and SEMs for the various cases as listed in the preceding section. Where appropriate, group means were tested with one-way ANOVA followed by post-hoc evaluation using Fishers LSD test.

Results

Retrograde tracing

Injections of FG into medial, middle and lateral parts of the RRF and the PAGvl (Fig. 1) produced remarkably similar distributions of labeled neurons within the prefrontal cortex, striatopallidum, preoptic area, lateral hypothalamus, zona incerta, mesopontine tegmentum and medial brainstem reticular formation, but, most prominently, in the extended amygdala (EA) and a network of forebrain and brainstem structures with which it is reciprocally interconnected (Figs. 2–4; Tables 2 and 3). Labeling observed following such injections was mainly ipsilateral, but at more caudal levels possessed a more robust contralateral representation, culminating in the mesopontine tegmentum at about half the ipsilateral density. The basic organization is mapped in Figure 3 from a case with an FG injection in the middle part of the RRF (see Fig. 1B for injection site). A few significant deviations from the basic pattern are noted in the following text and some of these are illustrated in Figure 4. Widespread forebrain and brainstem retrograde labeling was observed following many control injections of FG listed in the Materials and Methods section, but in no case, with the exception of the caudolateral SNc (see below and Table 2), did the pattern approximate that consistently observed after injections in the RRF and PAGvl.

An abundance of retrogradely labeled neurons was present in the ipsilateral and, to a lesser extent, contralateral, frontal cortex, including the frontal association, prelimbic, infralimbic, dorsal peduncular and dysgranular insular areas and the claustrum (Fig. 3A–D). Less dense labeling was present in the ipsilateral anterior cingulate cortex (Fig. 3B and C) and fewer labeled neurons, scattered within the dorsolateral convexity of the frontal cortex were observed only after injections in the lateral RRF (Fig. 4B and C).

Sparse to moderate retrograde labeling was observed in the accumbens, mainly in the rostral pole and ventromedial part of the shell (Figs. 3B and C), except following lateral RRF injections, which produced very dense labeling not only in the accumbens core, rostral pole and ventromedial and lateral shell (Fig. 4A), but also a ventralmost tier of the caudate-putamen extending from the lateral ventricle to the external capsule (Fig. 4A–C). Labeled neurons in the caudate-putamen and globus pallidus (Fig. 4C), however, may have resulted from the nearly unavoidable involvement of the lateral part of the substantia nigra in lateral RRF FG injection sites (see Fig. 1C–C’). To test this idea, we made a number of control PHA-L injections into the ventral caudate-putamen and failed to produce significant anterograde labeling in the RRF or PAGvl (not shown).

Sparse labeling was observed in the subcommissural ventral pallidum after PAGvl and RRF injections (Fig. 3D and 4B). Significantly, the density of labeling at the edges of and adjacent to the ventral pallidum exceeded that in the ventral pallidum itself. This was true both above the VP, in relation to the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), a component of the extended amygdala (Shammah-Lagnado et al., 2001), and below it, in a stratum of immunocytochemically extended amygdala (EA)-like (also Shammah-Lagnado et al., 2001) tissue that intervenes between the ventral pallidum and diagonal band complex (Fig. 3D). Dense labeling of IPAC extended over the caudal dorsolateral convexity of the subcommissural pallidum into the so-called ventral pocket of the striatum (Figs. 3D and E). The BST, notably excepting its medial division, contained dense labeling (Figs. 2A and A’ and 3E and F) and an additional band of dense labeling stretching across the sublenticular region (Figs. 3E–G) merged with dense labeling in the medial division of the CeA (Fig. 3H). In contrast, the medial amygdaloid nucleus (Fig. 3I), like the medial division of the BST (Fig. 3E and F), contained no retrogradely labeled neurons. See Discussion for notes on BST subnuclei and other subdivisions.

Labeling in the ventral pallidum, EA surrounding it, and BSTl-v merged caudally with remarkably dense labeling in the lateral (Figs. 3E–G) and medial (Figs. 3E and 4C and F) preoptic areas. The parvicellular division of the paraventricular nucleus of the hypothalamus, which is abundantly interconnected with EA, contained many retrogradely labeled neurons mostly in its caudal part (Figs. 3G and H and 4G and H). The lateral hypothalamic area, including the paraventricular nucleus, which has strong reciprocal interconnections with various EA structures (Goto and Swanson, 2004), contained dense numbers of FG labeled cells following injections into the RRF (Figs. 3I and 4D) and PAGvl (Fig. 4I). Dense accumulations of labeled neurons were present in the mediobasal and periventricular hypothalamus following FG injections into the PAGvl (Fig. 4G and H) but not RRF (Fig. 3F–I and 4B and C).

In the diencephalon, the lateral habenular nucleus (LHb) contained many retrogradely labeled neurons, while the medial habenula was empty (Fig. 3I). In the caudal diencephalon, dense accumulations of labeled neurons were present in the thalamic parafascicular nucleus, zona incerta and central gray following all of the RRF and PAGvl injections (Figs. 3I and J and 4D and I).

Labeled neurons were scattered fairly densely through much of the mesencephalic and rostral pontine tegmentum after injections of FG into the RRF, but the most prominent pontomesencephalic labeling occupied the PAGvl (Fig. 3K). Conversely, injections of FG into the PAGvl produced labeling in RRF neurons. Injections into the medial RRF produced dense bilateral labeling in the deep layers of the superior colliculus (not shown), which was not observed after FG injections into the PAGvl or other parts of the RRF and thus may reflect projections to nearby structures, such as the rostromedial tegmental nucleus (Jhou et al., 2009). Moderate to dense labeling was present in the substantia nigra reticulata (Fig. 3K), compacta (Fig. 3L) and, to a lesser extent, VTA (Fig. 3K) after all of the PAGvl and RRF injections. Some retrogradely labeled neurons located in dopaminergic structures following injections of FG into the RRF and PAG exhibited co-localized tyrosine hydroxylase immunoreactivity, but most did not. Dense retrograde labeling was present in the parabrachial nucleus (Fig. 3N), which is abundantly interconnected with the extended amygdala, and additional moderate labeling in the pons and medulla was most dense in the medial, gigantocellular part of the reticular formation (Figs. 3O–P). Labeled neurons were also present in the nucleus of the solitary tract, with which forebrain extended amygdaloid structures are interconnected (Fig. 4E and J). All RRF and PAGvl injections produced labeled neurons in the deep cerebellar nuclei (Fig. 3O).

The retrograde labeling results are further summarized in Tables 2 and 3, which also show data for comparative purposes from some VTA cases archived from a previous study (Geisler and Zahm, 2005) and some injections into the substantia nigra compacta (see, e.g., Fig. 1E and E') and reticulata. Table 2 gives estimates of the numbers of retrogradely labeled neurons produced in various structures by different injections and documents the abundance of retrograde labeling in structures comprising and interconnected with the EA following FG injections in the RRF, PAG and SNc. Table 3 provides the numbers of *structures* labeled by each of the injection sites and shows that all parts of the complex produce robust retrograde labeling in many cortical and subcortical sites, with the RRF and PAGvl ranking as the most diversely innervated of the midbrain dopaminergic structures.

Anterograde tracing

Extended amygdala and preoptic area—Following injections of PHA-L into structures comprising the EA, including the BST, CeA and sublenticular region, robust, topographically organized anterograde labeling was observed in the ventral midbrain dopaminergic complex, mainly in the RRF and PAGvl, but also in the VTA and SNc. The general pattern was for labeled axons to descend through the lateral hypothalamus in the medial forebrain bundle and, upon reaching the VTA and SNc, turn lateralward as variously compact bundles (Fig. 5A and A' and 6A and B) enroute to the RRF (Fig. 5B and B' and 6C and D) and PAGvl (Fig. 5C and C' and 6D). Depending upon where in the BST the injection site was, labeled projections varied with regard to topography and how terminally branched and 'varicose', i.e., beaded in appearance, were the axons (Fig. 5A', B' and C'). Terminal branching and axonal varicosities, a.k.a. "puncta", are a reflection of the functional potency of labeled axons (see *Methodological considerations* in the Discussion). In view of this revealing feature of PHA-L and BDA labeling, all of the included maps of anterogradely transported PHA-L were created by using the Neurolucida hardware-software platform to plot exclusively axonal varicosities, i.e., puncta (arrowed structures in Figure 5A'), such that non-varicose, presumably mainly non-synaptic, parts of labeled axons are excluded from the maps.

The projection from the BST to the midbrain dopaminergic complex is topographically organized. PHA-L injections into BSTl-d (Fig. 7A) produced bundles of labeled axons that turn lateralward quite rostrally in the VTA, thus achieving a position in the lateral part of the

SNc at a rostral level. Mapping of puncta proximal to the RRF after BSTl-d injections revealed mainly passing fibers with few axonal varicosities (Fig. 8A and B). A plexus of highly varicose labeled axons was present in the RRF, mainly in its lateral part (Fig. 8C). The PAGvl also contained numerous highly varicose labeled axons following injections in BSTl-d (Fig. 8D). Injections of PHA-L slightly more ventral in BSTl (Fig. 7B) produced labeling in axons that turn lateralward further caudal in the VTA, spread more broadly (i.e., medialward) within the RRF (Fig. 8G), and, particularly in the SNc and to some extent the VTA, were more varicose (Fig. 8E and F). Far ventral BST injections of PHA-L (Fig. 7C) completed a trend toward more widespread varicose labeling in the SNc, RRF, PAGvl and VTA (Fig. 8I–L), although the density of labeled puncta in the VTA consistently remained significantly less than in the SNc, RRF and PAGvl. Interestingly, injections of PHA-L into the lateral preoptic area (LPO) beneath the BST (Fig. 7D) produced a pattern of anterograde labeling in the ventral mesencephalon similar to that seen after far ventral BST injections, albeit more diffuse and substantially more concentrated in the VTA (Fig. 8M–P). Indeed, the pattern seen in the RRF and VTA after LPO injections could be imagined as a culmination of the transformations in the labeling pattern associated with moving injection sites more ventrally in BSTl.

Injections of PHA-L into CeAm (Fig. 9, injection sites shown in Fig. 7E and F) produced a pattern of anterograde labeling in the VTA, SNc, RRF and PAGvl that is topographically organized and nearly identical to that observed after BSTl injections. Injections into CeAm-l produced labeled fibers that, like BSTl-d injections, turn lateralward early, have few puncta in the SNc and almost none in the VTA, but ramify extensively in the lateral RRF and, albeit less so, in the PAGvl (Fig. 9A–C). In contrast, injections into CeAm-m produced a pattern of labeled fibers (Fig. 9D–F) resembling that observed after BSTl-v injections, i.e., widespread varicose labeling in the SNc, RRF and PAGvl (Fig. 8I–L) with moderate labeling of puncta in the VTA. Like the pattern of terminations in the midbrain after ventral BST injections resembled that of the LPO, the pattern observed after CeAm-m injections resembled that seen following injections into the LH, which lies just medial to CeAm-m and gives rise to projections to the midbrain dopaminergic complex much like those from LPO (see below under *Prelimbic cortex and lateral hypothalamus*).

One available injection of PHA-L into the sublenticular part of the EA (case 95109, Fig. 10A, inset) produced broadly dispersed, robust, varicose anterograde labeling in the SNc, medial and lateral RRF (Fig. 10B and B'), PAGvl (Fig. 10C and C') and modest varicose labeling in the VTA (Fig. 10A and A').

With the aid of the Neurolucida hardware-software platform, we were able to count the numbers of puncta plotted and derive measures of areal density in the various parts of the midbrain dopaminergic complex following injections of anterograde tracer in the BST, CeA, LPO and VP (see Quantitation of anterogradely labeled axonal varicosities in Materials and Methods). The data, shown in Figure 11, reveal that central extended amygdala structures (only BST and CeA projections were quantitated) richly innervate the RRF and PAGvl, in contrast to the LPO, which more strongly innervates the VTA, and ventral striatopallidum, which strongly innervates the VTA and medial SNC.

In contrast to the central division of the EA, PHA-L injections into the medial division of the EA produced no anterograde labeling in the ventral mesencephalon. For example, PHA-L injected into the medial division of the BST produced massive labeling in the mediobasal hypothalamus, but none in the midbrain dopaminergic complex (cases 99120 and 06029, data not shown). Likewise, an injection of PHA-L into the medial amygdaloid nucleus produced no anterogradely labeled fibers in the midbrain (case 00060, not shown), consistent with another published report (Canteras et al., 1995).

Ventral pallidum—Injections of PHA-L into the medial part of the subcommissural ventral pallidum produced robust anterograde labeling of fibers mainly in the VTA and, to a much lesser extent in medial parts of the RRF. Few fibers traversing the SNc were observed following such injections. In contrast, PHA-L injections into more lateral parts of the subcommissural ventral pallidum produced dense labeling of the lateral VTA, dorsomedial SNr and overlying parts of the SNc. Many varicose and non-varicose axons fibers traversed the SNc. The former contributed to scattered tufts of terminal axonal branching within the SNc. Slight to moderate labeling of axons in the RRF was observed following VP injections and a few labeled fibers were seen in the PAGvl.

A number of cases were available in which the anterogradely and retrogradely transported tracer, biotinylated dextran amine (BDA), was injected into the ventral pallidum. In such cases, BDA presumably is transported anterogradely not only in axons of ventral pallidal neurons that picked up the tracer at the injection site, but also in the axon collaterals of back-filled striatal neurons (Chen and Aston-Jones, 1998; Zahm, 2006), of which many project to the ventral mesencephalon (Lu et al., 1998). In these cases, dense, highly varicose anterograde labeling, presumably reflecting overlapping pallidomesencephalic and striatomesencephalic projection fibers, was prominent in the VTA, SNc and SNr (Fig. 12A–B), by comparison to the modest numbers of labeled axons in the RRF (Fig. 12C) and PAGvl (Fig. 12D). One injection site, for the case illustrated in Figure 12, is shown in Fig. 7G and only that case was quantitated and included in Fig. 11.

Lateral habenula—Injections of PHA-L (cases 070142, 08002, 08004 and 08011, not shown) produced moderate, varicose labeling in the VTA and a strong patch of varicose labeling in the RMTg, which abuts the VTA caudally. In addition, sparse varicose anterograde labeling was observed in the SNc, RRF and PAGvl.

Zona incerta—A PHA-L injection into the zona incerta (case 95108, Fig. 13A) produced a very dense plexus of labeled axons in the mesencephalic tegmentum mainly dorsal to the VTA and SNc (Fig. 13B). Although the interpretation of case 95108 would have benefited from demonstration of tyrosine hydroxylase immunoreactivity to precisely indicate boundaries of the RRF (estimated as outlined in Fig. 13C–E), the territories known to contain dense tyrosine hydroxylase immunoreactivity, such as the VTA, SNc and RRF, were mainly, but not entirely, avoided by labeled axons in case 95108. An extensive plexus of labeled fibers was present in the lateral PAG, but the ventrolateral PAG (outlined in Fig. 13F), where numerous tyrosine hydroxylase immunoreactive neurons are present (Fig. 1D and 1D inset), was relatively devoid of such labeled fibers.

Prelimbic cortex and lateral hypothalamus—Following a PHA-L injection into either the deep layers of the prelimbic cortex (cases 04062, and 04066, not shown) or lateral hypothalamus (cases 96049, 96108, and 96199, not shown), a moderately dense plexus of labeled varicose axons pervaded the VTA. A significant number of these veered laterally to pass into the SNc, mostly as fibers of passage lacking numerous axonal varicosities in the case of PrL, but moderately varicose after LH injections. PrL injections produced a moderately dense plexus of labeled axons located just rostral to the lateral RRF, mainly in the posterior intralaminar thalamic nucleus. For both injection sites, sparse projections containing immunoreactive PHA-L were present throughout the RRF, however, and these appeared to be part of a more widespread sparse distribution of labeled fibers occupying much of the mesencephalic reticular nucleus. A modest plexus of labeled axons was present in the PAGvl following injections of PHA-L into the PrL and LH.

Ventral mesencephalon—A number of cases with PHA-L injections into sites in the ventral midbrain within and around the dopaminergic complex were made (see Materials

and Methods for cases). Most injections produced some varicose anterograde labeling in the midbrain dopaminergic complex and this tended to be greater for sites located within the complex. Very dense anterograde labeling was present in the VTA and SNc and, to a lesser extent, the RRF and PAG following injections in the RMTg (see also Jhou et al., 2009).

Discussion

The combination of results from retrograde and anterograde axonal tracing described herein reveals that projections from the EAc stand out as predominant among manifold inputs to the RRF and PAGvl (Fig. 3, Table 2). Robust RRF- PAGvl inputs also arise in the hypothalamic paraventricular nucleus, lateral hypothalamus, paraventricular nucleus, parabrachial nucleus and nucleus of the tractus solitarius (Sol), which happen to be structures with which the core structures of the EA, the BST, i.e., the subnucleus EA and CeA, are most extensively interconnected. Indeed, Aston-Jones and colleagues (Mejias-Aponte et al., 2009) reported that the peak density of noradrenergic projections from Sol to the ventral mesencephalon is in the RRF. This interconnectivity of EAc structures with the RRF-PAGvl supports the conclusion that the RRF and PAGvl together represent a sector of the midbrain dopaminergic complex serving prominently as a node for outputs from EAc. These results complement earlier published findings by Hasue and Shammah-Lagnado (2004) indicating that projections originating in the PAGvl, including the dorsal raphe nucleus embedded within it, and the RRF, contain the majority of tyrosine hydroxylase-immunoreactive neurons providing innervation to the EA (see also Table in Zahm and Trimble, 2008).

Methodological considerations

The retrograde labeling results reported here are subject to potential confounds, such as uptake of tracer by damaged and undamaged fibers-of-passage and spread of injected tracer to unintended structures, that have been discussed at length in our recent papers (Geisler and Zahm, 2005; Jhou et al., 2009). However, in our present and recent evaluations of inputs to ventral mesencephalic structures (e.g., Geisler and Zahm, 2005; Jhou et al., 2009), we almost always observed that structures with retrograde labeling, when targeted by anterogradely transported PHA-L, do exhibit some varicosity-decorated labeled axons in the structures of interest. Sometimes sparse inputs to the target structure comprise but part of a more widely distributed projection, as do, e.g., prefrontal cortical, lateral preoptic area (Fig. 8M-P) and lateral hypothalamic projections to the RRF/PAGvl shown in the present study. Alternatively, input from a strongly innervated adjacent structure may encroach into the target structure, e.g., as the robust ZI projection to the dorsolateral PAG invades the PAGvl (Fig. 13F). It should also be acknowledged that the uptake of FG by axons is vigorous (Brog et al., 1993; Geisler and Zahm, 2005), such that FG uptake even by a sparse projection may give rise to abundant retrogradely labeled neurons. Conversely, a relatively small number of retrogradely labeled neurons, such as are observed, e.g., in the mesopontine rostromedial tegmental nucleus following FG injections into the VTA and SNc, may give rise to a very dense projection field (Jhou et al., 2009). Thus, because purely spurious retrograde labeling is rare in our experience, we conclude that the capacity of retrograde tracing to reveal *all* of the *potential* afferents of a structure has value that cannot be overestimated. The possibility that retrogradely labeled sites might later be shown to lack, or, alternatively, have particularly robust inputs to the midbrain dopaminergic complex enhances rather than negates the usefulness of a comprehensive map of the retrograde labeling (as in Fig. 3).

Complementary anterograde tracing is an essential control, however, and, in the present investigation, we used new and archived cases with injections of anterograde tracer into numerous structures, illustrating a selection of those that we regarded as more likely to have projections critical to the validity of the conclusions suggested by the retrograde tracing.

One example of an apparently spurious retrograde labeling result disclosed by anterograde labeling was the avoidance of the VTA, SNc and RRF by the projection labeled after a PHA-L injection in the ZI (Fig. 13), despite prominent retrograde labeling observed in the ZI following retrograde tracer injections in the RRF (Figs. 3J and 4D and I). Consistent with considerations mentioned earlier, the observed retrograde labeling in ZI likely reflects the sparse but detectable input of ZI to the RRF/PAGvl and uptake of tracer by ZI axons enveloping the VTA, SNc and RRF (Fig. 13).

In describing the anterograde tracing results, we distinguished what we refer to as ‘varicose’ labeled axons, i.e., ones exhibiting a beaded appearance, from those lacking such apparent differentiation. Insofar as axonal swellings typically, although not always, exhibit synapses when viewed with electron microscopy (e.g., Loopuijt and Zahm, 2006; Omelchenko and Sesack, 2009; 2010), varicose projections may be conditionally regarded as likely to reflect functional, synaptic relationships.

Finally, we noted that the retrograde labeling observed within the VTA, SNc, RRF and PAGvl mainly, although not entirely, involved neurons lacking tyrosine hydroxylase immunoreactivity, suggesting a mainly non-dopaminergic local circuitry. It should be pointed out in this regard, that combining DAB and nickel DAB to generate brown and black immunoperoxidase products, respectively, as was done in the present study, is not an entirely satisfactory way to show such double labeling, due to a tendency of these chromogens to obscure each other. Further investigation is necessary to better address the issue of the phenotypes of the interconnections among the different structures comprising the midbrain dopaminergic complex. The importance of this interconnectivity is highlighted by much early evidence identifying the rostral mesencephalon as a critical interface linking cognitively generated directives originated in the forebrain with a hindbrain-spinal cord circuitry autonomously capable of supporting of differentiated, coordinated postural and motoric function (e.g., Ferrier, 1876/1966; Hinsey et al., 1930; Bard and Macht, 1958; Harris, 1958, for reviews).

Extended amygdala

Two divisions of the EA, central (EAc) and medial (EAm), have been described (de Olmos and Ingram, 1972; Alheid and Heimer, 1988; Heimer et al., 1991a; Heimer and Alheid, 1991; de Olmos and Heimer, 1999). EAc consists of the CeA, BSTl and hodologically similar sectors in the sublenticular region and stria terminalis that extend between them. EAc is characterized by robust outputs to the hypothalamic paraventricular nucleus, lateral hypothalamus, parabrachial nucleus and nucleus of the solitary tract, consistent with an involvement in the central regulation of autonomic function. EAm comprises the medial amygdala, medial division of the BST and hodologically similar parts of the sublenticular region and stria terminalis that extend between them. EAm projects robustly to the mediobasal hypothalamus, consistent with a role in the regulation of neuroendocrine function.

Earlier studies from numerous laboratories showed that EAc projects strongly to the dopaminergic midbrain tegmentum (e.g., Krettek and Price, 1978; Price and Amaral, 1981; van der Kooy et al., 1984; Holstege et al., 1985; Gonzalez and Chesselet, 1990; Rosen et al., 1991; Zahm et al., 1999; Fudge and Haber, 2000; Shammah-Lagnado et al., 2001; Gastard et al., 2002; Zahm, 2006). The present investigation has revealed that these projections possess a previously unrecognized degree of specificity for RRF-PAGvl. Indeed, we have shown here that EAc projections involve the VTA and rostromedial SNC to a much lesser extent than the RRF-PAGvl, mainly passing through the VTA and rostromedial SNc with few varicosities and negligible branching before diverging laterally to densely innervate the caudolateral SNc and the RRF and PAGvl. The present results also indicate that EAm does

not project to the midbrain dopaminergic complex, consistent with other reports (e.g., Canteras et al., 1995; Dong and Swanson, 2004b).

It should be noted that some authors maintain that EAc-VTA projections are robust (Fudge and Haber, 2000, in the monkey). Furthermore, presumably monosynaptic activations of VTA neurons following stimulation of the BST have been reported in the rat (Georges and Aston-Jones, 2001; 2002; Dumont and Williams, 2004; Massi et al., 2008; Jalabert et al., 2009). Tracer injections positioned in the ventral part of the BST in the present study produced a distinctly broad and uniform spread of tracer within the RRF and moderate anterograde labeling in the VTA, much as Swanson and colleagues also observed a 'diffuse' projection pattern after injection of PHA-L into the fusiform nucleus (a ventral BSTI structure) as compared to the oval nucleus, which has a more dorsal position in the BSTI (Ju and Swanson, 1989; Dong et al., 2001). Ventral parts of the BST merge without detectable boundaries into the dorsal part of the preoptic-rostral hypothalamic area, which has very strong projections to the VTA (Phillipson, 1979; Swanson et al., 1984; Simerly and Swanson, 1988; Zahm et al., 2001; Geisler and Zahm, 2005; 2006; Reynolds et al., 2006; present results) that are in significant part glutamatergic (Geisler et al., 2007; Geisler and Wise, 2008). Monosynaptic excitations recorded in the ventral mesencephalon following stimulation of the ventral BST may reflect the existence there of a transitional region possessing characteristics of both BST and the preoptic-rostral hypothalamic region, which would help to reconcile the functional findings not only with the relative scarcity of EAc-VTA projections that we observe, but also with literature indicating that few BST neurons are glutamatergic (McDonald et al., 1989; Takayama and Miura, 1991; Sun and Cassell, 1993; Geisler and Zahm, 2007).

A comment is required regarding BST nomenclature. Based on developmental and cytoarchitecture considerations, Swanson and colleagues subdivided the BST into anterior and posterior *divisions* (Ju and Swanson, 1989; Ju et al., 1989) and further designated up to 20 named BST *areas* and better differentiated *nuclei*, some of which they considered together as *groups* referencing putative functional interrelationships (e.g., Dong et al., 2000; 2001; Dong and Swanson, 2003). The distributions of immunocytochemical and histochemical markers and input-output patterns in the BST, however, are not well constrained by Ju and Swanson's (1989) nuclear boundaries, which were predicated mainly on neuron clustering in Nissl preparations. It is true that Dong and colleagues have described a number of connectional differentiations of BST subnuclei in their extensive series of studies with the anterograde tracer PHA-L (Dong et al., 2000; 2001; Dong and Swanson, 2003; 2004a; b; 2006a; b; c), but, viewed overall, those results tend as much to support the conspicuous mediolateral differentiations repeatedly identified by others (e.g., Krettek and Price, 1978; Ricardo and Koh, 1978; Bleier et al., 1979; Schwaber et al., 1982; Weller and Smith, 1982; Sofroniew, 1983; van der Kooy et al., 1984; de Olmos et al., 1985; Holstege et al., 1985; Alden et al., 1994; Alheid et al., 1995) that form the conceptual basis for distinguishing EAc and EAm (refs cited above). Lateral BST injections in the Dong et al. series of papers produced robust anterograde labeling in EAc structures and the midbrain dopaminergic complex (e.g., Dong et al., 2000; 2001; Dong and Swanson, 2003; 2004a; 2006c). For instance, they (Dong et al., 2000) reported a dense BST-nigral projection from Ju and Swanson's (1989) juxtacapsular nucleus that much resembles the robust innervation of the SNc that we report arises in BSTI and CeAm (see also Gonzalez and Chesselet; Vankova et al., 1992). Conversely, their medial injections (e.g., Dong and Swanson, 2004b), like ours (data not shown) into their anterodorsal area (a structure in BSTm) showed dense interconnections only with EAm structures and lacked projections to the midbrain tegmentum, just as they reported for the medial amygdaloid nucleus (Canteras et al., 1995), another EAm component. Dong and colleagues also studied several nuclei far ventral in the BST complex with projections reminiscent of neither (or both) EAc or EAm (Dong and

Swanson, 2006a; b). These structures are not readily categorized, but their projections may reflect the transitional character of this ventral region interposed between the main part of the BST and the preoptic-rostral hypothalamic continuum. In view of these considerations, we have described our findings in the BST with reference to its mediolateral and dorsoventral dimensions.

Striatopallidum

Voluminous basal ganglia inputs to the substantia nigra compacta arise in the caudate nucleus, putamen and globus pallidus (Nauta and Mehler, 1966; Grofova and Rinovik, 1970; Beckstead et al., 1979; Gerfen, 1985; Jimenez-Castellanos and Graybiel, 1989). Those to the VTA originate principally in the nucleus accumbens (Swanson and Cowan, 1975; Conrad and Pfaff, 1976; Powell and Leman, 1976; Zahm et al., 2001; Geisler and Zahm, 2005) and ventral pallidum (Groenewegen et al., 1993). Nauta et al. (1978), however, observed that fibers projecting from the nucleus accumbens to the VTA veer lateralward to also innervate much of the SNc, prompting Nauta and Domesick (1984) to maintain that the projection “is distributed in greatest volume to the medial half of the pars compacta”.....and.... “impulses from the nucleus accumbens could be thought to affect the nigral innervation, and hence the functional state, of the entire medial half of the striatum.” A projection from the lateral part of the accumbens continues strongly into the lateral RRF and, more sparsely, the PAGvl (Nauta et al., 1978; Zahm and Heimer, 1993; Usuda et al., 1998; Shammah-Lagnado et al., 2001). Indeed, we can confirm with some unpublished PHA-L cases very strong projections from the lateral shell of the Acb to the lateral part of the RRF and quite modest projections from medial Acb to the medial and middle RRF. Injections of PHA-L into the ventral pallidum in the present and an earlier published study (Groenewegen et al., 1993) also produced prominent anterograde labeling in the VTA that turns lateralward to traverse and innervate less robustly the SNc and RRF and, to a yet lesser extent, the PAGvl, further reinforcing the idea that the ventral striatopallidum innervates primarily the VTA, but, less densely, the entire midbrain dopaminergic complex.

Septum and lateral preoptic area (LPO)

The lateral septum, a main terminus of outputs from the hippocampus conveyed mainly via the subiculum (Jakab and Leranth, 1995), has meager projections to the ventral midbrain and these are essentially limited to the VTA (Geisler and Zahm, 2005). In contrast, the LPO, the main recipient of outputs from the lateral septum (Staiger and Nurnberger, 1991; Jakab and Leranth, 1995; Risold and Swanson, 1997; Zahm, 2006), gives rise to projections to the VTA that are among the densest it receives (Zahm et al., 2001; Geisler and Zahm, 2005; 2006; Geisler et al., 2007). The functional potency of the pathway from the LPO to the VTA is reflected in the observation that locomotion is robustly stimulated when the LPO is unilaterally infused with bicuculline, a GABA A receptor antagonist that activates by disinhibition (Reynolds et al., 2006, see also Sinnamon, 1993). The present data indicate that the LPO also provides a moderately dense innervation of the SNc, RRF and PAGvl, such that the influence of the LPO could be conveyed throughout the entire midbrain dopaminergic complex. To our knowledge specific functional implications of LPO projections to SNc, RRF and PAGvl have not been addressed experimentally.

Functional considerations

That the dominant input to the RRF-PAGvl comes from the EAc and numerous autonomic effector sites in the hypothalamus and brainstem that are EAc projection targets (e.g., Zahm et al., 1999; Dong et al., 2000; 2001; Dong and Swanson, 2004a; b; 2006c; Goto and Swanson, 2004; Gastard et al., 2005) suggests that the RRF-PAGvl subserves the synthesis of a broad variety of emotional/motivational, orienting, bodily maintenance and homeostatic responses to which the EAc is well known to contribute (e.g., Applegate et al., 1983; Pascoe

and Kapp, 1985; LeDoux et al., 1988; Davis et al., 1997; Davis and Shi, 1999; Nader et al., 2001; Sullivan et al., 2004; Wilensky et al., 2006; Price et al., 1987; Rizvi et al., 1991; Goto and Swanson, 2004). Indeed, a convincing case for linkage of the CeA and PAGvl precisely in this manner has been established (Rizvi et al., 1991), based in part on the intimate interconnectivity of the CeA and PAGvl and in part on the demonstration by Bandler and colleagues that stimulation of PAGvl projections to the ventral medulla elicits both behavioral and autonomic components of defensive/aversive responses (Carrive et al., 1987; 1988; 1989; Bandler and Carrive, 1988). Along similar lines, reportedly strong non-dopaminergic downstream projections from the RRF provide an anatomical link consistent with RRF modulation of brainstem somatomotor and visceromotor structures (von Krosigk and Smith, 1991; von Krosigk et al., 1992). Electrical and chemical stimulation of the RRF elicits forepaw and orofacial movements in cats and rats (Arts et al., 1998; Arts and Cools, 1998; 1999, 2000) and a projection from CeA to the SNc/RRF in rats has been implicated in Pavlovian responding (Gallagher et al., 1990; Hall et al., 2001; Lee et al., 2005; 2006; 2008; El-Amamy and Holland, 2007). In addition, evidence has been reported for an involvement of CeA interconnections with mainly the caudolateral SNc in the modulation of associative learning (Holland et al., 2000; Gallagher and Holland, 1994; Han et al., 1997; Lee et al., 2005; Holland and Gallagher, 2006).

How the activity of ascending ‘mesotelencephalic’ neurons is modulated by afferents is at present poorly understood, but certainly must in some way reflect how the afferents are organized within the midbrain dopaminergic complex. Clearly one would want to know the extent to which various afferent systems synaptically contact dopaminergic neurons, but where this has been addressed with electron microscopy (e.g., Groenewegen et al., 1994; Omelchenko and Sesack, 2005; 2006; 2009; 2010; Omelchenko et al., 2009), inputs invariably were shown to terminate on dopaminergic and non-dopaminergic neurons, which creates difficult issues requiring more sophisticated quantitation than has so far been brought to bear on the question. While it seems likely that some extended amygdala projections directly contact dopaminergic neurons, this remains to be shown. Conversely, we observed many PHA-L labeled puncta within the confines of the midbrain dopaminergic complex but relatively far from dopaminergic neurons, consistent with an innervation that is distributed among multiple cell types.

Concluding remarks

The present study reveals that each of Alheid and Heimer’s (1988) cortico-subcortical functional-anatomical macrosystems is mainly interconnected with a particular sector of the midbrain dopaminergic complex. That is to say, these data encourage the conceptualization of a midbrain dopaminergic complex in which *dominant* sources of inputs to its classically designated divisions - VTA, SNc and RRF (here regarded as complexed with the PAGvl) – comprise mainly projections from distinct groups of deep telencephalic nuclei. The VTA is innervated by ventral striatopallidum and the septal-preoptic system, SNc by dorsal striatopallidum and the RRF-PAGvl by the extended amygdala. The present data also reinforce a complementary concept - that so-called “limbic” afferents, such as those arising in the septal-preoptic system, ventral striatopallidum and extended amygdala, reach and thus presumably contribute to information processing in all parts of the midbrain dopaminergic complex, including the substantia nigra pars compacta, as has been advocated by others previously (e.g., Nauta et al., 1978; Gonzales and Chesselet, 1990; additional authors cited in the Introduction). We suspect that the interaction of these two aspects of organization underlies the tremendous versatility and subtlety with which the activity of neurons comprising the midbrain dopaminergic complex appears to be modulated.

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List of Abbreviations

ac	anterior commissure
AcbC	accumbens core
AcbRp	accumbens rostral pole
AcbSh	accumbens shell
AH	anterior hypothalamic area
AI	agranular insular cortex
Aq	cerebral aqueduct
BDA	biotinylated dextran amine
BSTl	bed nucleus of stria terminalis, lateral division
BSTl-d	BSTl, dorsal part
BSTl-i	BSTl, intermediate (mid-dorsoventral) part
BSTl-v	BSTl, ventral part
BSTm	bed nucleus of stria terminalis, medial division
BSTv	bed nucleus of stria terminalis, ventral division
CeA	central nucleus of the amygdala
CeAm	medial division of the central nucleus of the amygdala
CeAm-l	CeAm, lateral part
CeAm-m	CeAm, medial part
CeAl	lateral division of the CeA
Cg	cingulate cortex
Cl	claustrum
CN	deep cerebellar nuclei
CPu	caudate-putamen
CPuvl	ventrolateral caudate putamen
DI	dysgranular insular cortex
DP	dorsal peduncular cortex
EAc(SL)	central division of extended amygdala, sublenticular part
EPN	entopeduncular nucleus
FG	Fluorogold
f	fornix
FrA	frontal association cortex
FS	fundus striati

Gi	gigantocellular reticular nucleus
GI	granular insular cortex
GP	globus pallidus
ic	internal capsule
IL	infralimbic cortex
InG	intermediate gray layer of the superior colliculus
IPAC	interstitial nucleus of the posterior limb of the anterior commissure
IPN	interpeduncular nucleus
LH	lateral hypothalamic area
LHb	lateral habenula
MHb	medial habenula
LS	lateral septum
LO	lateral orbital cortex
LPO	lateral preoptic area
M	motor cortex
MHb	medial habenula
ml	medial lemniscus
MPO	medial preoptic area
MRN	mesencephalic reticular nucleus
MS	medial septum
mt	mammillothalamic tract
NOS	nitric oxide synthase
oc	optic chiasm
ot	optic tract
Pa	paraventricular nucleus of the hypothalamus
PAG	periaqueductal gray substance
PAGvl	ventral and lateral parts of the periaqueductal gray substance
PBI	parabrachial nucleus, lateral division
Pe	periventricular hypothalamus nucleus
Pf	parafascicular nucleus
PH	posterior hypothalamic area
PHA-L	<i>Phaseolus vulgaris</i> -leucoagglutinin
PPTg	pedunculopontine tegmental nucleus
PrL	prelimbic cortex
PSTh	parasubthalamic nucleus
PvP	posterior part of the thalamic paraventricular nucleus

RN	red nucleus
RRF	retrotrubral field
RRFc	caudal part of RRF
RRFl	lateral part of RRF
RRFm	medial part of RRF
sm	stria medullaris
SNC	substantia nigra compacta
SNCd	dorsal tier of SNC
SNr	substantia nigra reticulata
Sol	nucleus of the tractus solitarius
VDB	vertical limb of the diagonal band
VO	ventral orbital cortex
VP	ventral pallidum
MTA	ventral tegmental area
xscp	decussation of the cerebellar peduncle
ZI	zona incerta
3	third ventricle

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Figure 1.

Micrographs illustrating Fluorogold (FG) injection sites in the medial (A), middle (B) and lateral (C-C'') retrorubral field (RRF), ventrolateral periaqueductal gray (PAGvl, D and D inset) and lateral substantia nigra compacta (SNc, E and E'). The sections were processed to exhibit both FG and tyrosine hydroxylase (A-D) or neurotensin (E and E') immunoreactivities, which illustrate the extent of the ventral mesencephalic dopaminergic complex comprising the ventral tegmental area (VTA), substantia nigra compacta (SNc) and retrorubral field (RRF). In A through D, TH immunoreactivity is brown and the FG injection site black. In E and E' neurotensin immunoreactivity is black and the FG injection site brown. A and B show injection sites relatively confined to the medial and middle RRF,

respectively. C-C'' shows an injection site in the lateral RRF that minimally involves the SNc. D inset reveals by symmetry that the PAGvl injection site (D) occupies a part of the periaqueductal gray that contains numerous TH-immunoreactive neurons (brown stained neurons), indicative of the dopaminergic district A10dc. E and E' illustrate an FG injection site in the SNc (E) that minimally involves the RRF (E'). Case numbers are provided in the upper right corners of the leading panel for each case to facilitate identification of cases shown in subsequent illustrations and discussed in the text. Additional abbreviations: aq – cerebral aqueduct; IPN – interpeduncular nucleus; ml – medial lemniscus. Scale bar: 1 mm in A, B, C-C'', D and E-E'; 200 μ m in D inset.

Figure 2.

Micrographs illustrating retrogradely labeled neurons in the bed nucleus of stria terminalis (BST, A and A'), medial division of the central nucleus of the amygdala (CeAm, B and B') and hypothalamic paraventricular nucleus (Pa, C and C') of case 10197 resulting from an injection of FG into the lateral retrorubral field shown in Fig. 1C-C'. Panels A', B' and C' are enlargements of the boxes in corresponding panels A, B and C. Retrogradely labeled neurons (some examples indicated by arrows) exhibit black nickel-enhanced DAB immunoperoxidase product. Note that the labeling in the CeAm (B and B') is continuous with that in the medial preoptic area (MPO) and lateral preoptic area (LPO). The sections were counterstained with nitric oxide synthase immunoreactivity shown in brown to help

differentiate structures within the sections. Additional abbreviations: AH – anterior hypothalamus; BSTl – bed nucleus of stria terminalis, lateral division; BSTm – bed nucleus of stria terminalis, medial division; CeAl – central nucleus of the amygdala, lateral division; CPu – caudate-putamen; EPN – entopeduncular nucleus; GP – globus pallidus; LH – lateral hypothalamic area; to – optic tract; VP – ventral pallidum. Scale bar: 1 mm in A, B and C; 200 μ m in A', B' and C'.

Figure 3.

Map of retrograde labeling following an injection of Fluorogold into the middle (case 06166) of the retrorubral field. The injection sites is shown in Figure 1B and panel L. Each dot represents a retrogradely labeled neuron. For abbreviations, see list.

Figure 4.

Maps of retrograde labeling following injections of Fluorogold (FG) into the lateral retrorubral field (RRFI, case 10197, panels A–E') and ventrolateral periaqueductal gray (PAGvl, case 10099, panels F–J). The injection sites are shown in Figures 1C–C'' and 1D, respectively. Each dot represents a retrogradely labeled neuron. Additional abbreviations: AcbC – accumbens core, AcbSh – accumbens shell, BSTl – bed nucleus of stria terminalis, lateral division, BSTm – bed nucleus of stria terminalis, medial division, CeAm – medial division of the central nucleus of the amygdala, CeAl – lateral division of the central nucleus of the amygdala, CPu – caudate-putamen, EAc(SL) – central division of extended amygdala, sublenticular part, GP – globus pallidus, MPO – medial preoptic area, Pa – hypothalamic

paraventricular nucleus, Pe – periventricular nucleus, Pf – parafascicular nucleus, PSTh – parasubthalamic nucleus, Sol – nucleus of the tractus solitarius, ZI – zona incerta.

Figure 5.

Micrographs illustrating the distribution of anterogradely labeled axons in the midbrain dopaminergic complex in case 06042 (A-A') in which an injection of PHA-L (black injection site) was placed in the lateral division of the bed nucleus of stria terminalis (BST, inset in A) and case 06060 (B-C') with a PHA-L injection in the lateral division of the BST at a slightly more ventral position. White dots indicate the positions of PHA-L impregnated neurons marking the injection sites. A', B' and C' are enlargements of the respective boxes in A, B and C. In case 06042 a rostral section through the midbrain dopaminergic complex (A and A') exhibits dense anterograde labeling in the substantia nigra compacta (SNc) mostly in the form of non-varicose presumably passing fibers, although some varicosities

(puncta) are visible (arrows). Little anterograde labeling is visible in the ventral tegmental area (VTA) in this case at this level. In case 06060, anterograde labeling spreads homogeneously through the retrorubral field (RRF, B and B') and is more varicose (a few examples are arrowed). The ventrolateral periaqueductal gray substance (PAGvl) also contains a moderately dense plexus of varicose labeled fibers (C and C'). Additional abbreviations: ac – anterior commissure, EAc(SL) – central division of extended amygdala, sublenticular division; f – fornix; IPAC – interstitial nucleus of the posterior limb of the anterior commissure; IPN – interpeduncular nucleus; xscp – crossing of the superior cerebellar peduncle. Scale bar: 1 mm in A, B and C; 100 μ m in A', B' and C'.

Figure 6.

Micrographs illustrating case 06042 (also shown in Fig. 5) in sections processed for tyrosine hydroxylase (brown reaction product) and PHA-L (black reaction product) immunoreactivity, which reveals that anterograde labeling following the large PHA-L injection shown in the inset in Fig. 5A is present mainly within the midbrain dopaminergic complex. Additional abbreviations: PAGvl – ventrolateral periaqueductal gray; RRFc – caudal part of the retrotrubral field. Scale bar: 1 mm.

Figure 7.

Diagram showing PHA-L injection sites (A–F) and a BDA injection (G) in cases discussed in the text and illustrated in figures 8 (A–D), 9 (E, F) and 12. Two levels are shown for each injection. Case numbers are given on the more rostral sections of the pairs. Dots indicate PHA-L impregnated neurons, which are thought to give rise to the labeled projection.

Abbreviations: BSTI-d, BSTI-i and BSTI-v – bed nucleus of stria terminalis, lateral division, dorsal, intermediate and ventral parts, respectively; CeAm-l and CeAm-m – central nucleus of the amygdala, lateral and medial parts, respectively; LPO – lateral preoptic area; VP – ventral pallidum.

Figure 8.

Diagrams illustrating anterograde labeling associated with PHA-L injections at dorsal (99052 BSTl-d), intermediate (06042 BSTl-i) and ventral (06043 BSTl-v) parts of the lateral division of the bed nucleus of the stria terminalis (BSTl-d, BSTl-i and BSTl-v, respectively) and in the lateral preoptic area (09025 LPO). The injection sites are shown in Fig. 7A–F. Broken lines circumscribe the tyrosine hydroxylase-rich midbrain dopaminergic complex, these having been circumscribed from adjacent serial sections processed to illustrate TH immunoreactivity. Sections processed for TH immunoreactivity were unavailable for case 09052 (A–D). Note the increasing medialward spread of anterograde labeling in the RRF and particularly into the VTA with more ventral injections and that the anterograde labeling

after ventral BST injections most resembles that observed following injections into the LPO, particularly with respect to the VTA. For abbreviations, see list.

Figure 9.

Diagrams illustrating anterograde labeling associated with PHA-L injections in the lateral (A–C) and medial (D–F) parts of the medial division of the central nucleus of the amygdala (CeAm-l and CeAm-m, respectively). The injection sites are shown in Fig. 7E and F. Broken lines circumscribe the tyrosine hydroxylase-rich midbrain dopaminergic complex, having been circumscribed from adjacent serial sections processed to for TH immunoreactivity. Sections processed for TH immunoreactivity were unavailable for case 00061 (A–C). Note the increasing medialward spread of anterograde labeling in the RRF and particularly the VTA with more medial injections and that the anterograde labeling after

CeAm-m injections more resembles that observed following injections into the LPO (see Fig. 8M–P). For abbreviations, see list.

Figure 10.

Micrographs illustrating case 95108 in which an injection of PHA-L into the central division of the extended amygdala in the sublenticular region [EAc(SL)] resulted in abundant anterogradely labeled axons in the midbrain dopaminergic complex. Numerous labeled fibers were present in the ventral tegmental area (VTA, A and A'), including in a substantial number of varicose labeled axons. Labeled fibers were also observed in the substantia nigra compacta (SNc in A), retrorubral field (RRF in B and B') and lateral and ventrolateral parts of the periaqueductal gray substance (PAGvl in C and C'). For abbreviations, see list. Scale bar: 1 mm in A, B and C; 100 μ m in A', B', and C'; 2.5 mm in the inset.

Figure 11.

Graph showing results measuring the densities of labeled axonal varicosities, or “puncta” in the ventral tegmental area (VTA), substantia nigra compacta (SNc), retrorubral field (RRF) and ventral and lateral sectors of the periaqueductal gray substance (PAGvl) following injections of PHA-L at dorsal (BSTd), intermediate (BSTi) and ventral (BSTv) levels of the bed nucleus of stria terminalis, lateral (CeAm-l) and medial (CeAm-m) part of the medial division of the central nucleus of the amygdala, lateral preoptic area (LPO) and ventral pallidum (VP). The VP was injected with biotinylated dextran amine (BDA) rather than PHA-L. Data were evaluated by a one way ANOVA followed by Fisher’s LSD post-hoc test only in groups of 3 or more. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Figure 12.

Diagrams illustrating case 94052 in which an injection of BDA into the ventral pallidum (see Figure 7G for injection site) resulted in robust anterograde labeling in the ventral tegmental area (VTA in A and B) and substantia nigra compacta (SNc in A and B). A lesser amount of anterograde labeling was present in the retrorubral field (RRF in C and D) and PAGvl (D). Broken lines circumscribe the tyrosine hydroxylase-rich midbrain dopaminergic complex, having been circumscribed from adjacent serial sections processed for TH immunoreactivity.

Figure 13.

Micrographs illustrating case 95108 in which an injection of PHA-L into the zona incerta (ZI) resulted in dense retrograde labeling in the midbrain tegmentum that largely avoided the midbrain dopaminergic complex shown at the level of the ventral tegmental area (VTA in B), retrorubral field (C, D and E) and periaqueductal gray (PAGvl in F). The white dots in A indicate the position of neurons impregnated with PHA-L immunoreactivity. Broken lines designate the dorsal/dorsolateral margin of the VTA in B and approximate boundaries of the retrorubral field (C, D and E) and ventrolateral periaqueductal gray (PAGvl in F). Note Anterograde labeling within the PAGvl diminishes ventrally where many of the tyrosine

hydroxylase immunoreactive neurons that project to the central division of the extended amygdala are located. For abbreviations, see list.: Scale bar: 1 mm.

Table 1**Primary Antibodies**

<u>Antiserum</u>	<u>Immunogen</u>	<u>Source (cat. no.)</u>	<u>Working dilution</u>
Rabbit polyclonal anti-Fluorogold (FG)	Fluorogold itself	Bioscience Research Reagents, a division of Millipore, Temecula, CA (AB153)	1:5000 – 1:10,000
Rabbit polyclonal anti-neurotensin (NT)	Synthetic neurotensin (ELYENLPRRPYIL) conjugated to bovine thyroglobulin with glutaraldehyde	Immunostar, Hudson, WI (20072)	1:5000 – 1:10,000
Rabbit polyclonal anti-nitric oxide synthase (Nos)	Amino acids 251–270 of nitric oxide synthase (GDNDRVFNDLWGKDNVPVILC) conjugated to keyhole limpet cyanin	Sigma Chemical Company, St. Louis, MO (N7155)	1:5000 – 1:10,000
Rabbit polyclonal anti- <i>Phaseolus vulgaris</i> leucoagglutinin (PHA-L)	<i>Phaseolus vulgaris</i> -leucoagglutinin (E & L) itself	Vector Laboratories, Burlingame, CA (AS-2224)	1:5000 – 1:10,000
Mouse monoclonal anti-tyrosine hydroxylase (TH)	Tyrosine hydroxylase from PC12 cells	Bioscience Research Reagents, a division of Millipore, Temecula, CA (MAB318)	1:5000 – 1:10,000

Table 2

Retrograde labeling following injections of Fluoro-Gold

Retrogradely labeled structures	Injection sites					
	VTA	RRF		PAGvl	SN	
		med	mid	lat	c	r
<u>Cortex</u>						
frontal association	0	xxx	xx	xxx	xxx	0
medial orbital	0	x	x	xx	xxx	0
lateral orbital	0	xx	x	xx	xxx	0
prelimbic	xxx	xxx	xx	xxx	xxx	0
infralimbic	xxx	xxx	xx	xxx	xxx	0
dorsal peduncular	xxx	xxx	xx	xx	xxx	0
anterior cingulate	xx	xxx	xx	xx	xx	0
agranular insular	x	xxx	xxx	xxx	xxx	xx
claustrum	xx	xx	x	xxx	xx	x
dysgranular insular	0	0	x	xxx	xx	0
granular insular	0	0	x	xxx	xx	0
second somatosensory	0	0	0	xx	0	xxx
primary somatosensory	0	0	0	xx	0	xxx
second motor	0	0	0	xx	0	xx
primary motor	0	0	0	xx	0	xx
<u>Basal ganglia</u>						
accumbens rostral pole	xxx	xx	0	xxx	xx	x
accumbens medial shell	xxx	xxx	x	0	x	0
accumbens lateral shell	x	x	xx	xxx	xxx	x
accumbens core	xxx	0	x	xxx	0	xxx
ventral pallidum	xxx	xxx	xx	xx	xxx	0
caudate-putamen (mainly v and vl)	x	0	0	xxx	xx	xxx
globus pallidus	xx	x	0	xxx	xx	xxx
entopeduncular nucleus	0	xx	x	0	xx	0
subthalamic nucleus	0	0	0	xxx	0	xxx

	Retrogradely labeled structures				Injection sites			
	VTA	RRF			PAG-vl	SN		
		med	mid	lat		c	r	
substantia nigra compacta	xxx	xx	xx	xxx	xxx	xxx	xx	xx
substantia nigra reticulata	x	xx	xx	xxx	xxx	xxx	xx	xx
<u>Septum and extended amygdala</u>								
lateral septum	xxx	xx	xx	o	x	o	o	o
medial septum-diagonal band	xxx	xx	xx	o	x	o	o	o
bed nucleus of stria terminalis, med-d	xx	xxx	xxx	xxx	x	xxx	o	o
bed nucleus of stria terminalis, lat-d	x	x	x	xxx	xxx	xxx	o	o
bed nucleus of stria terminalis, med-v	xx	xxx	xxx	xxx	x	xx	o	o
bed nucleus of stria terminalis, lat-v	x	xxx	xxx	xxx	xxx	xx	o	o
marginal nucleus of Shu et al. (1988)	o	o	o	xxx	o	xxx	xxx	xxx
sublenticular extended amygdala	xxx	xxx	xxx	xxx	xxx	xxx	xx	xx
anterior amygdaloid area	xxx	xxx	xxx	xxx	xxx	xxx	xx	xx
central amygdaloid nucleus, lat	xx	xxx	xxx	xxx	o	xxx	x	x
central amygdaloid nucleus, med	xx	xxx	xxx	xxx	xxx	xxx	xx	xx
<u>Hypothalamus</u>								
median preoptic nucleus	xx	xx	x	o	xxx	o	o	o
lateral preoptic area	xxx	xxx	xx	xxx	xxx	xxx	o	o
medial preoptic area	xxx	xxx	xxx	xxx	xxx	xxx	o	o
lateral hypothalamic area	xxx	xxx	xxx	xxx	xxx	xxx	o	o
paraventricular nucleus	xx	xx	xx	xxx	xxx	xx	o	o
posterior hypothalamic area	xxx	xxx	xx	o	xxx	x	o	o
central gray	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx
supramammillary nucleus	xxx	xx	o	o	xxx	o	o	o
mammillary body	x	xx	o	o	o	o	o	o
<u>Thalamus and epithalamus</u>								
lateral habenula	xxx	xxx	xx	xx	xxx	xxx	o	o
parafascicular nucleus	xxx	xxx	xxx	xxx	xxx	xxx	o	o
ventromedial nucleus	x	xxx	xx	xx	o	xx	xx	xx
posterior intralaminar nucleus	xx	xxx	x	xx	xxx	xx	xx	xx

Retrogradely labeled structures	Injection sites					SN	
	VTA	RRF	PAGvl	c	r		
<u>Other structures (ordered rostrocaudally)</u>							
zona incerta	xxx	xxx	xxx	xxx	xx		
parasubthalamic nucleus	x	o	xxx	xxx	o		
ventral tegmental area	xx	x	xx	xxx	xx		
interpeduncular nucleus	nd	x	o	o	o		
rostromedial tegmental nucleus	xxx	x	xx	xxx	xx		
superior colliculus, deep layers	xx	xxx	xxx	xxx	xx		
periaqueductal gray	xxx	xxx	xxx	xxx	xx		
deep mesencephalic nucleus	xx	xxx	xxx	xxx	xx		
dorsal raphe	xxx	xxx	xx	xxx	xx		
pedunculopontine tegmental nucleus	xxx	xxx	xxx	xxx	xx		
laterodorsal tegmental nucleus	xxx	xxx	xx	xxx	x		
median raphe	xxx	xx	o	xx	o		
parabrachial nucleus	xx	xxx	xxx	xxx	xx		
cuneiform nucleus	xxx	xx	xxx	xxx	xx		
pontine reticular nucleus, oralis	xx	xxx	xx	xxx	x		
pontine reticular nucleus, caudalis	x	xxx	xx	xxx	x		
principal nucleus V, ventrolateral (contralat)	o	xx	x	xx	o		
<u>Evaluated cases</u>	99039	08024	06108	05103	10098	05104	05096
	99060		06157	05105	10099	05106	05095
			06166	10090			
				10091			
				10096			
				10097			

Representative injection sites are illustrated in Figure 1. o = absent or trace retrograde labeling reflecting 0–5 labeled cells/structure. + = 6 to 15 labeled neurons/section. ++ = 16–50 labeled neurons/section. +++ = more than 50 labeled neurons/section/structure. This list references labeling of ipsilateral structures following the indicated injections, listed in approximate rostrocaudal sequence. Representative contralateral labeling is illustrated in Figure 3. Abbreviations: c – compacta; d – dorsal; lat – lateral; lat-d – lateral division, dorsal part; lat-v – lateral division, ventral part; med – medial; med-d – medial division, dorsal part; med-v – medial division ventral part; mid – middle; PAG – periaqueductal gray; RRF – retrorubral field; SN – substantia nigra; v – ventral; vl – ventrolateral; VTA – ventral tegmental area.

Table 3

Summary of Table 2¹

Injection site	Number of retrogradely labeled structures				
	xxx	xx	x	o	Total
RRF lat	43	15	1	8	60
PAGvl	39	14	4	11	57
RRF med	35	15	7	11	57
SNC	32	18	3	15	53
VTA	28	15	10	14	53
RRF mid	22	16	13	17	51
SNr	9	20	6	32	35
					73

¹ Data are transposed from Table 2 such that columns 2–4 from the left list the numbers of retrogradely labeled structures with xxx, xx, x and o ratings following Fluoro-Gold injections in the listed Injection Sites. The column entitled “Total” gives the numbers of structures with labeling, i.e., the sum of the values in columns xxx, xx and x. The “x index” is equivalent to the value in column xxx multiplied by 3 plus that in column xx multiplied by 2 plus that in column x and provides a relative estimate of the overall magnitude of retrograde labeling for each injection site. The injection sites are listed in order of descending magnitude of the retrograde labeling that they produced. Abbreviations: dorsal, caudal; lat – lateral; mid – middle; RRF – retrorubral field; rvIPAG – rostral ventrolateral periaqueductal gray; SNC and SNr – substantia nigra compacta and reticulata; VTA – ventral tegmental area.