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COLLATERALIZATION OF MONOAMINE NEURONS: MESOTELENCEPHALIC DOPAMINE PROJECTIONS TO CAUDATE, SEPTUM, AND FRONTAL CORTEX[1]

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Abstract

Axonal collateralization of substantia nigra (SN) and ventral tegmental area (VTA) neurons was studied in the rat with the multiple retrograde labeling technique using fluorescent dyes. Injections of nuclear yellow, granular blue, and propidium iodide were made into the caudate-putamen, septum, and frontal cortex and cells were found to be single, double, and triple labeled in the SN-VTA. Cells in the VTA were predominantly single labeled with the three tracers and cells in the medial SN were predominantly double labeled with the tracers. These results suggest that mesotelencephalic neurons in the SN-VTA can be subdivided into two subpopulations on the basis of relative degree of collateralization. The SN axons appear to be highly collateralized to anatomically and functionally distinct telencephalic areas and the VTA axons appear to have a more singular one-to-one type of projection to the same forebrain regions.

The mesotelencephalic dopamine neuron system includes the projections of the dopamine cells of the ventral tegmental area (VTA) and substantia nigra (SN) to the forebrain. The term "mesotelencephalic" (Björklund and Lindvall, 1978; Moore and Bloom, 1978) implies a basic organization that modifies earlier concepts (Ungerstedt, 1971) of the separation of dopamine neurons into a "nigrostriatal" SN projection to the caudate-putamen and a "mesolimbic" VTA projection to the limbic structures of the basal forebrain. Recent anatomical studies (Fallon and Moore, 1976a, b, 1978a, b; Fallon et al., 1978a, b; Fallon, 1980) support the concepts implied in this terminology by demonstrating that the cells of the SN and VTA form a continuum with an organized topography of projections, in three planes, to the forebrain. A major finding in these studies was that the distinction between "nigrostriatal," "mesolimbic," and "mesocortical" dopamine neurons was not based on their inclusion into SN or VTA groups but rather based on their geometric position, especially in the dorsoventral plane, in the SN-VTA continuum. Further support and expansion of these concepts arose in reports from other laboratories (Domesick et al., 1976; Beckstead, 1976; Faull and Mehler, 1978; Nauta et al., 1978; Phillipson, 1979a, b, c; Chiodo et al., 1980; Groenewegen et al., 1980).

Despite the newly discovered segregation of SN dopamine cells by highly organized topographical principles, it appeared that there was some overlap in the origin of dopamine neuron projections to functionally and anatomically separate forebrain structures, such as the caudate-putamen, septum, amygdala, and frontal cortex (Fallon and Moore, 1978b). In fact, for a decade, there has been some evidence to suggest that individual dopamine cells might have axon collaterals that innervate separate pairs of structures, such as the caudate-putamen and central nucleus of the amygdala (Ungerstedt, 1971; Fallon and Moore, 1978b), the caudate-putamen and cingulate cortex (Lindvall et al., 1974), and the septum and prefrontal cortex (Lindvall et al., 1977). Recent physiological (Deniau et al., 1980) and anatomical evidence (Fallon, 1980; Fallon and Loughlin, 1981; Loughlin et al., 1981) suggests that some mesencephalic dopamine neurons do, indeed, innervate more than one forebrain structure.

In the present study, we investigated the collateralization of individual dopamine neurons of the SN-VTA to the caudate-putamen, septum, and prefrontal cortex. These structures were chosen for study because they are major forebrain targets of dopamine neurons, they receive an innervation from overlapping areas in the medial SN and VTA that contain dopamine neurons (Fallon and Moore, 1978b; Fallon, 1980), and they represent components of the functionally divergent striatal, limbic, and cortical forebrain systems. The analysis was made possible by the recent introduction of the multiple retrograde labeling techniques using fluorescent tracers with different spectral emission properties (Kuypers et al., 1977, 1979; Bentivoglio et al., 1980a, b). This technique has been used successfully for tracing pathways within the mesostriatal dopamine neuron system (van der Kooy,

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1979). Preliminary results of these studies have been published in recent abstracts (Fallon, 1980; Fallon and Loughlin, 1981; Loughlin et al., 1981).

Materials and Methods

Experimental animals and surgical procedures

Forty male and female albino rats (Simonsen, Gilroy, CA) weighing 200 to 360 gm were used in the study. The animals were anesthetized with Nembutal (Abbott), 30 mg/kg, i.p., or ether (Mallinckrodt) and prepared for aseptic surgery. Injections were made in a Kopf stereotaxic apparatus.

Fluorescent tracers and injections

The technique used here is a modification of the method described by Kuypers et al. (1977, 1979) and Bentivoglio et al. (1980a, b). The fluorescent substances employed in the experiments included nuclear yellow (NY, Hoechst S 769121), granular blue (GB, 186/134, Friedrich-Alexander University), true blue (TB, 150/129), bisbenzimide (Bb, Hoechst 33258), and propidium iodide (PI, Sigma P-5264). Table I lists the range of concentrations in distilled water and the volume of each tracer that was used in the study.

For 30 of the 40 animals, the combination of tracers used was NY, GB, and PI. The injections were made into area 32 of the prefrontal cortex, septum, and medial caudate-putamen nucleus in a counterbalanced design such that nine combinations of tracers and forebrain targets were used. Half of the injections were made through 1-μl Hamilton syringes and half were made through glass micropipettes fitted to a nanoliter pump (W-P Instruments). The rate of injection ranged from 3 to 10 min/100 nl, with the average rate being 5 min/100 nl. Following an injection, the syringe or pipette was left in place for 5 min before removal from the brain. The rate of removal was approximately 1 mm/sec.

Survival times and perfusion

The survival times ranged from 10 to 36 hr. Routinely, survival times were 20 hr. Following survival times, the animals were anesthetized deeply with Nembutal, 40 mg/kg, i.p. They were perfused transcardially with 200 ml of 0.9% saline followed by 500 ml of 10% formalin/saline (equivalent to a 4% solution of paraformaldehyde) at a range of pH values from 5 (unbuffered) to 7.8 (buffered). We routinely used a pH of 7.8 because of the optimal fluorescence obtained.

TABLE I

Fluorescent tracers used in the study and the range of concentrations and volumes used

Tracer	Concentration		Volume	
	Range	Most Often Used	Range	Most Often Used
	%		nl	
Nuclear yellow (NY)	1–10	5	50–300	100
Granular blue (GB)	5–10	5	50–500	150
True blue (TB)	5–10	5	50–500	150
Bisbenzimide (Bb)	5–10	10	50–300	200
Propidium iodide (PI)	1–5	3	50–200	80

Tissue processing

The brains were removed and postfixed for 2 to 14 hr in a 30% sucrose in 10% formalin/saline solution or cacodylate buffer (pH 7.2). The sections were cut at 30 μm on a Leitz freezing microtome, washed in distilled water or stored in cacodylate buffer, mounted onto acid-cleaned slides, and coverslipped with Entellan (Merck 1960). The slides were analyzed immediately and then stored in the dark at 4°C.

Microscopy and analysis

The location of the injection sites and retrogradely labeled cells was plotted onto standardized drawings of coronal sections of the rat brain. The sections were viewed under a Zeiss microscope fitted with epi-illumination and Zeiss fluorescence filter packages. Excitation fluorescence at 360 to 380 nm (Zeiss 487702-9902) was used for viewing NY, Bb, GB, and TB. Excitation fluorescence at 510 to 560 nm (Zeiss 487714-9900) was used for viewing PI. Each section and field of view was analyzed for single or double labeling by switching the filters back and forth. It is possible, however, to detect that a cell has been labeled with NY, GB, and PI by examination under the 360- to 390-nm filter alone because NY labels the nucleus yellow and GB labels the cytoplasm with blue granules. A faint orange PI fluorescence also can be seen, and later verified, by switching to viewing with the 550-nm filter. The faint orange PI fluorescence seen with the wide band blue 360- to 390-nm filter can be eliminated by inserting a narrow band blue filter (Zeiss 467800 BG 23 Blau filter) into the light beams exiting the fluorescence filter package. This is useful for taking black and white photographs of NY- and GB-labeled cells, especially when PI fluorescence is unwanted.

Photography

Black and white photographs were taken on 35-mm format Tri-X film (Kodak, ASA 400), and color photographs were taken on Ektachrome 200 film (Kodak, ASA 200). Normally, exposure times ranged from 5 to 30 sec. The photographic parameters for the film are detailed in Table II. Black and white prints were made with FP-1-1 Agfa-Gevaert Radioprint paper. The prints were made directly from color slide film. The red insensitivity of the paper can be used to advantage if it is desirable to reduce the orange-red PI fluorescence in the black and white prints.

Controls

Injections of distilled water or saline were made into the caudate-putamen of three rats to determine what levels of background fluorescence were due to tissue processing, microscopy, and aldehyde perfusion. The appearance of autofluorescence in neural and non-neural structures was examined. Combined injections of the tracers were made into the caudate-putamen, septum, frontal cortex, and surrounding structures to determine if the tracers had the same efficacy of retrograde labeling in the SN-VTA and to ascertain the general visual appearance of double and triple labeled cells.

TABLE II

Exposure times for taking photographs

The ranges of exposures are given and mean exposure time is shown in parentheses. 35-mm film photographs were taken on a Zeiss Ultraphot II photomicroscope fitted with epi-illumination, a mercury HBO light source (50 W), a 12.5 ocular lens, and a × 1.25 optovar. Exposure times with × 4 and × 10 objectives are for injection sites or very dense retrograde labeling.

Objective Lens	Total Magnification	Tri-X (Black and White)		Ektachrome 200 (Color)	
		Blue Filter	Red Filter	Blue Filter	Red Filter
		sec		*sec*	
× 4	× 62	5–20 (10)	40–70 (60)	15–50 (30)	20–60 (35)
× 10	× 156	5–10 (7)	20–50 (40)	10–30 (15)	20–40 (30)
× 16 oil	× 250	1–5 (3)	10–40 (30)	5–15 (10)	20–30 (25)
× 25 oil	× 390	1–5 (3)	10–50 (30)	3–10 (5)	10–30 (20)
× 40 oil	× 625	5–10 (7)	20–50 (30)	10–30 (15)	10–30 (15)

Results

Analysis of method and parameters. Complete parametric analyses have not been reported for the fluorescence retrograde tracing techniques. The technique has been modified in different laboratories for tracing specific pathways and neuronal systems. For the mesotelencephalic dopamine neuron systems, we have found that the optimal results were obtained with the following procedure. NY, GB, and PI provide the best combination of tracers because they are readily available, are visible and distinguishable in one cell, and contain a fluorophor that does not fade dramatically with storage or exposure time. The GB (and TB) tracer, however, has the most rapid fading during ultraviolet exposure, remaining visible for only 30 to 60 sec. Fortunately, fading appears to be as great, or greater, for background fluorescence so that the signal-to-noise ratio sometimes actually improves with long exposure to ultraviolet light. In order to obtain similar sized injection sites, different volumes or concentrations of NY, GB, and PI must be used. For a 2-mm^3 injection site, the injection volumes would be NY, 100 nl; GB, 150 nl; and PI, 80 nl. The number of retrogradely labeled cells in the SN-VTA using equally sized injection sites varies, however, and although it appears that NY and GB produce similar results, there are at least twice as many cells retrogradely labeled with PI. With similar sized injections of horseradish peroxidase (HRP) into the same structures (Fallon and Moore, 1978b), the number and position of retrogradely labeled cells in the SN-VTA are similar to results with NY and GB but only about half that of PI. For this reason, we most often utilized NY and GB as an optional pair for assessing collateralization. The optimal and convenient survival time for a 7- to 10-mm neural projection is 20 hr. Shorter times (less than 18 hr) result in weaker labeling, and significantly longer times (greater than 36 hr) result in glial uptake and occasional secondary neuronal uptake in cells adjacent to retrogradely labeled cells. This results in glial and neuronal "halos" forming around intensely labeled cells. This problem is greatest with NY and least with GB. To reduce uptake in cannula tracts, glass micropipettes are used for the injections. Anterograde fiber labeling rarely produces problems of interpretation, and only GB or TB are easily recognized in anterogradely labeled axons. Due to safety considerations, cacodylate buffer is not used routinely in tissue processing although excellent results are obtained with its usage. Although sections need not be coverslipped for viewing under the microscope, there is improved visibility and storage life in coverslipped sections. If stored properly, usable photographs may be obtained a month after tissue processing. Delaying the cutting or mounting of brain sections for more than a few hours, however, does lead to increased background fluorescence and more diffuse and dimmer specific fluorescence. For best tissue quality, it is advisable to mount sections from water within 3 min of cutting to minimize damage due to distilled water and to analyze the tissue within 24 hr. The overall impression of the technique, when compared to other multiple labeling techniques employing autoradiography (^{3}H-lectin, ^{3}H-HRP), is excellent in terms of ease, economy, sensitivity, and reliability. As retrograde tracers, the fluorescent compounds compare favorably to HRP for similar reasons. These optimistic conclusions await extensive parametric analyses, controls, and statistical comparisons to verify their veracity.

Controls. Injections of distilled water into forebrain targets did not result in any specific fluorescent labeling and, when compared to cases where fluorescent tracers were used, the injections of water did not affect the background fluorescence or autofluorescence. Yellow-orange and orange-red autofluorescent granules were observed in some vascular and perivascular cells, but these are easily distinguished from the more homogeneous orange-red PI fluorescence with both the 360–390- and 550-nm filters. Some neurons, such as those of the mesencephalic nucleus of the trigeminal nucleus, however, do autofluoresce with a dim orange-red color similar to that of PI. This orange-red fluorescence seen with the 550-nm filter does not appear a pale orange with the 360- to 390-nm filter as does specific PI fluorescence. Therefore, this second type of nonspecific orange-red fluorescence can be differentiated from specific PI fluorescence. However, rigorous quantitative studies are needed to verify the subjective observation. Injections of mixtures of NY, GB, and PI into the caudate-putamen resulted in an unequal amount of retrograde labeling in the SN-VTA, even if the apparent injection sites were co-extensive. The descending order of efficacy for the number of cells labeled and the extent of labeling in the SN-VTA is PI, NY, and GB. This discrepancy could be adjusted by injecting the tracers in volume ratios of GB:NY:PI, 3:2:1. This was determined empirically and is used only as a gross approximation of the relative equivalence of retrograde labeling with the three tracers. Another, perhaps more satisfactory, method to equilibrate relative uptake was to inject the same volume of the three tracers (100 nl) and to vary the concentrations used.

Injection sites. Examples of the three types of injection sites are shown in Figure 1. The cases were considered to be useful when the injection sites were localized to the septum, area 32 of frontal cortex, and caudate-putamen, which included sites where dopamine axons have been shown to terminate (for review, see Lindvall and Björklund, 1978; Moore and Bloom, 1978). It is important to stress that the injection site, particularly in the caudate-putamen and septum, did not include fibers of passage destined for the frontal cortex. Dopamine fibers projecting to the frontal cortex travel ventral to the injection sites included in the present study; therefore, this possible confounding variable has been avoided. The size of the injection sites varied from 0.5 to 2.5 mm^3. If there was any spillage into adjacent areas, the cases were not considered useful. In the cases where the injections missed any of the three primary targets but were restricted to other dopamine recipient terminal areas, such as the nucleus accumbens or the nucleus of the diagonal band, the cases were analyzed and will be reported below.

Retrograde labeling in the SN-VTA. A photomicrograph of the SN-VTA is shown in Figure 2. The description of the VTA and SN has been given in detail in

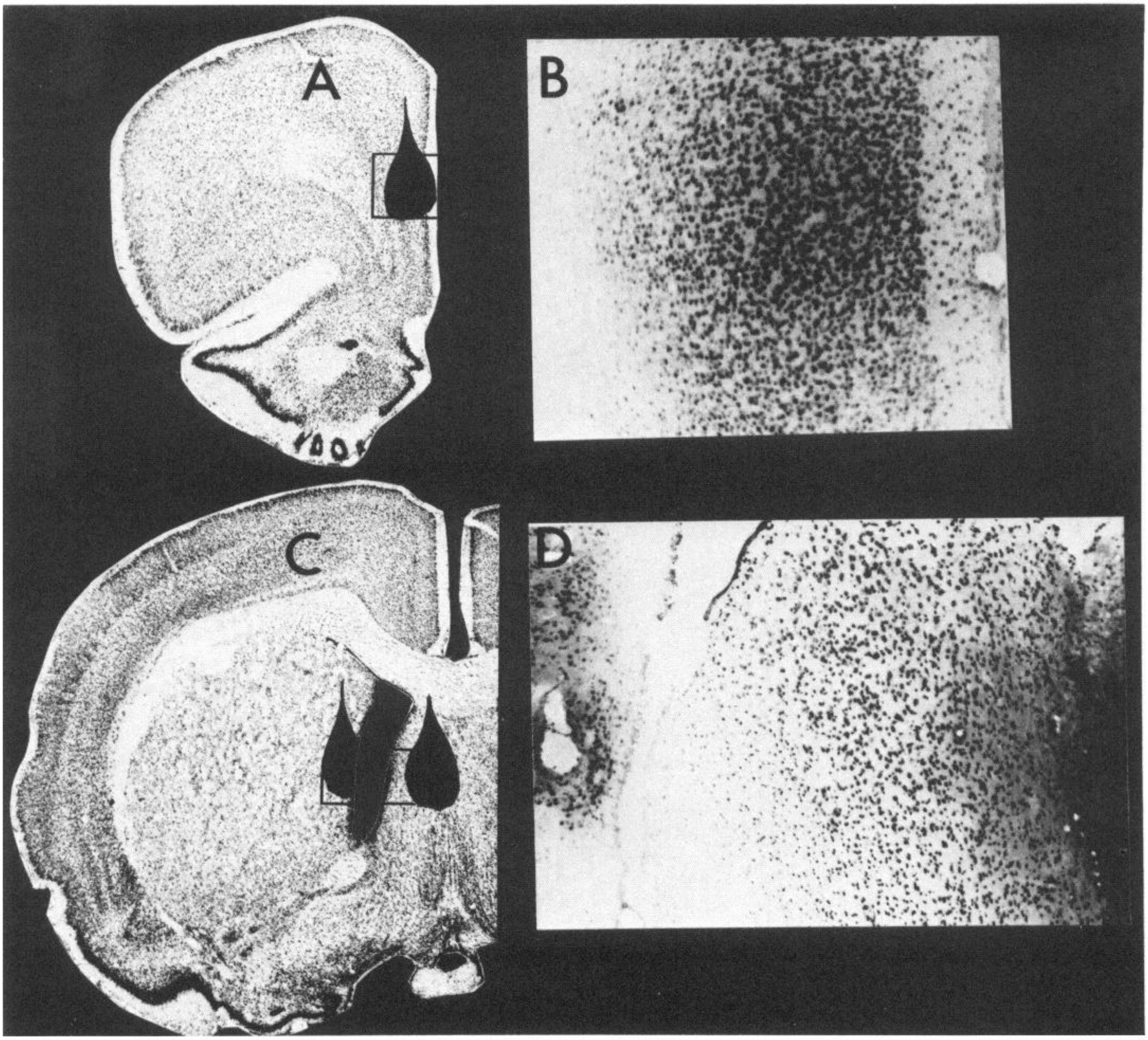

Figure 1. Photomicrographs of coronal sections of rat brain illustrating typical injection sites in the pregenual frontal cortex (*A* and *B*) and in the septum and medial caudate-putamen (*C* and *D*). *A*, Injection of PI into the frontal cortex. The *black area* illustrates the extent of the primary and secondary zone of injection. *B*, The *boxed area* in *A* is shown at higher magnification. *C*, Injection of GB (medial caudate-putamen) and NY (septum). *D*, Parts of both injections sites (see *boxed area* in *C*) in the caudate (*left*) and septum (*right*). The GB injection site appears less extensive than the NY injection site because of the dimmer fluorescence of the GB tracer and the spectral characteristics of the Ektachrome film and Kodak paper.

previous reports (Fallon and Moore, 1978b; Phillipson, 1979a, b). Of particular interest in the present report are neurons located in the ventrally placed nucleus paranigralis of the VTA and the adjacent sector of the medial SN. Following injections of NY, GB, and PI into the medial caudate-putamen, lateral septal nucleus, and deep layers of the pregenual sector of prefrontal cortex, cells retrogradely labeled with the three tracers are observed in the lateral part of nucleus paranigralis of the VTA and the ventral cells of the medial SN. The labeling is most numerous in the anterior half of the SN-VTA. Injections into the medial caudate-putamen resulted in more retrograde labeling in medial SN than nucleus paranigralis. Injections into the lateral septal nucleus resulted in more retrograde labeling in nucleus paranigralis than medial SN. Injections in the pregenual sector of the prefrontal cortex resulted in more retrograde labeling of dorsal cells in the nucleus paranigralis and medial VTA. There is, however, an overlap of retrogradely labeled cells in the SN-VTA following the three injections. Within this zone of overlap, there are cells that are labeled with one tracer, cells that are labeled with two tracers, and several cells that are labeled with all three tracers. There are cells of the SN-VTA, therefore, that collateralize and innervate all three terminal areas. Upon closer inspection, it is apparent that there is a difference between the SN and VTA in the number of cells that are single, double, and triple labeled. There are many double labeled, and a rare triple labeled cell in the SN following these injections; in fact, multiple labeling is a common feature in this area. The cells of the nucleus paranigralis, on the other hand, are rarely double labeled. This observation is particularly striking when three adjacent cells in the VTA are single labeled with NY, GB, and PI.

In cases where injections are made into the nucleus accumbens, prefrontal cortex, and septum, the same pattern emerges. Medial SN cells are usually double labeled and occasionally triple labeled, but VTA cells were almost always single labeled.

Discussion

The major finding of this study is that the ascending projections of the SN-VTA to the telencephalon can be subdivided on the basis of degree of axonal collateralization. The medial SN projection to the medial caudate-putamen, lateral septum, and pregenual sector of prefrontal cortex is characterized by extensive collateralization; that is, most of the cells send axonal branches to two, or all three, terminal areas. The VTA projection to the same terminal areas, on the other hand, is characterized by a more limited one-to-one relationship between VTA cell body and terminal field. Thus, VTA cells may be collateralized less extensively than SN cells.

This finding is in direct support of the recent physiological demonstration (Deniau et al., 1980) that VTA projections to the frontal cortex, septum, and nucleus accumbens arise from different cells. The present study extends these observations by demonstrating that, within the population of SN-VTA cells, there exist two types of cells that can be differentiated by a unique characteristic, that is, the degree of axonal collateralization. Our previous reports (Fallon and Moore, 1978b; Fallon et al., 1978b) stressed the concept that one major differentiating characteristic within the SN-VTA dopaminergic cell projections was that the dorsally placed cells of the SN projected to ventral forebrain limbic and allocortical structures and that the ventrally placed cells of the SN projected to the dorsally situated structures, such as the caudate-putamen. Thus, the dorsal cells of the SN were very similar to VTA cells in that they formed the "mesolimbic" component of the mesotelencephalic projections. These connectional similarities, which implied that the SN-VTA formed a medial-lateral continuum, were extended by the cogent cytoarchitectonic, Golgi, and connectional descriptions of Phillipson (1979a, b, c).

In the present report, it is proposed that there is a distinction between the ventral VTA and the immediately adjacent cells in the ventral tier of the medial SN. The distinction is in the specific way that the cells collateralize. Whether the dorsally placed SN cells are similar to the VTA cells in this respect is presently being investigated in this laboratory by making more ventrally placed injections into the olfactory tubercle and amygdala. If the dorsal SN and VTA are similar in that the cells are not highly collateralized, the concept of a dorsal SN-VTA "limbic" continuum distinct from a ventral SN "striatal" continuum (Fallon and Moore, 1978b; Fallon et al., 1978b; Phillipson, 1979a, b, c) would be further substantiated.

An important issue that could not be answered by the present study is whether the type of collateralization is correlated with the catecholaminergic nature of the SN-VTA cells. It is known that both dopaminergic and non-dopaminergic cells in the SN-VTA project to the telencephalon (Berger et al., 1978; Guyenet and Aghajanian, 1978; Simon et al., 1979 a, b; Deniau et al., 1980; Yim and Mogenson, 1980) and it would be of great interest, on both anatomical and functional grounds, to know if collateralization is related to the type of transmitter present in the cell. Since the cells in the ventral tier of the medial SN are dominated overwhelmingly by dopaminergic cells, it can be stated that many of the highly collateralized cells described in this study probably contain dopamine. It appears quite certain now that the differences in the SN-VTA neurons are not necessarily only between dopaminergic and non-dopaminergic cells. Physiological studies (Bunney, 1979; for review, see Chiodo et al., 1980; Maeda and Mogenson, 1980) and pharmacological studies (Browder et al., 1981) also distinguish two types of dopaminergic cells in the SN-VTA. It is possible, for example, that the highly collateralized SN cells described here could be the same type of dopaminergic neuron that we (Fallon et al., 1978b) described earlier as the ventral mesostriatal type, that Guyenet and Aghajanian (1978) described as Type 1, that Chiodo et al. (1980) described as Type B, or that Browder et al. (1981) described as L-amphetamine insensitive. Likewise, the poorly collateralized VTA cells described here could be the dorsal mesolimbic (Fallon et al., 1978b), Type A (Chiodo et al., 1980), or L-amphetamine-sensitive (Browder et al., 1981) neurons described by the same authors. Furthermore, the dorsal cells are differentiated by a significant input from enkephalinergic neurons (Johnson et al., 1980). These proposed associations are of a highly speculative nature, but they demand intensive future investigation. The primary question as to the dopaminergic or non-

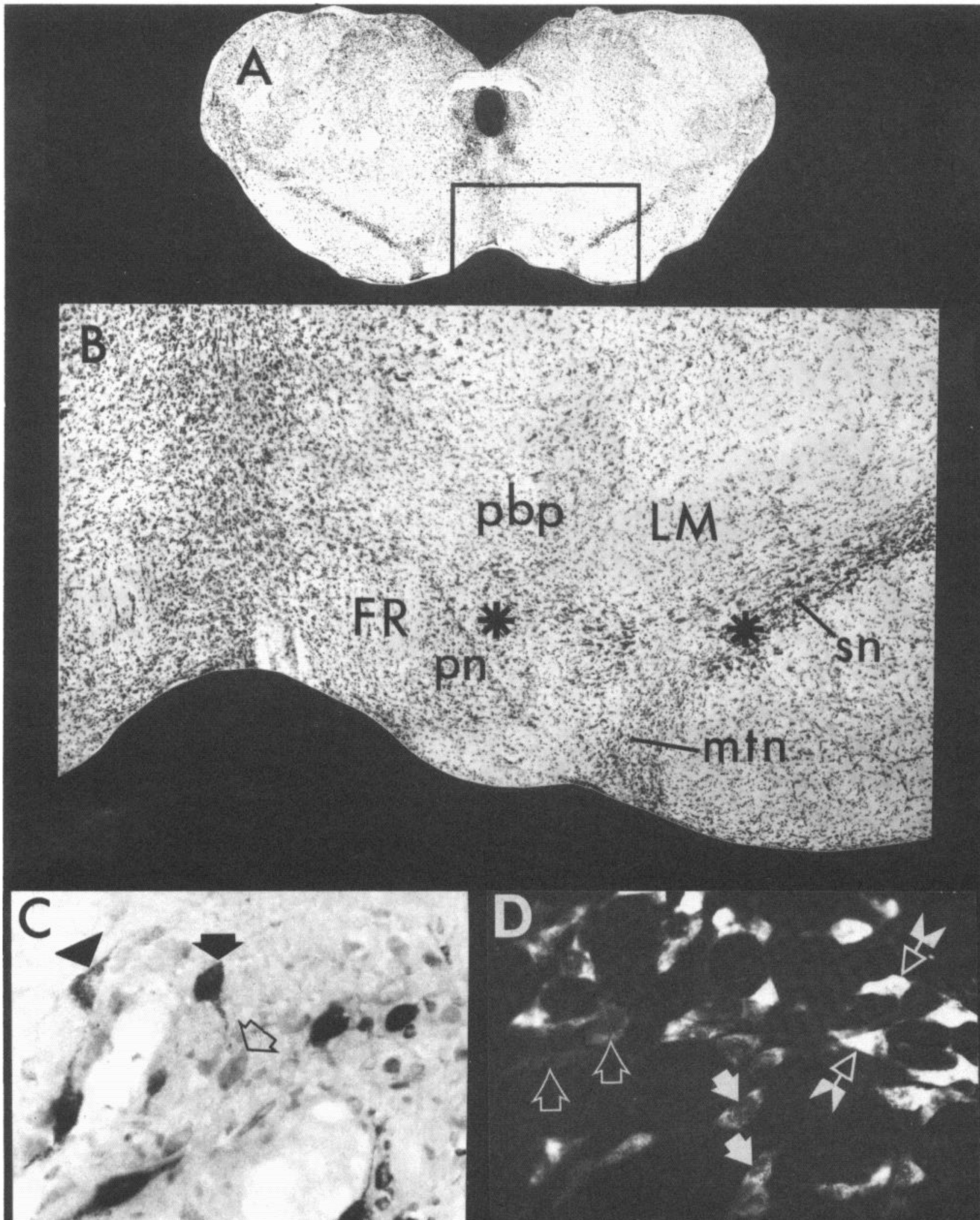

Figure 2. Photomicrographs of coronal sections of the rostral mesencephalon of the rat at the Konig and Klippel A 2180 level. *A*, Low power photomicrograph of the SN-VTA. The *boxed area* is represented in *B*. *B*, Medium power photomicrograph of the medial SN-VTA. Landmarks include the fasciculus retroflexus (*FR*), medial lemniscus (*LM*), medial terminal nucleus (*mtn*), substantia nigra pars compacta (*sn*), paranigral nucleus (*pn*), and parabrachialis pigmentosus nucleus (*pbp*). The *left asterisk* designates the area of the *pn* from which the photomicrograph in *C* was taken. The *right asterisk* in the *sn* designates the area from which the photomicrograph in *D* was taken. *C*, Photomicrograph taken from the *pn* showing single labeling of neurons

dopaminergic nature of the cells could be determined by combining the multiple fluorescent retrograde transport technique described here with the glyoxylic acid or paraformaldehyde-induced fluorescence techniques for endogenous monoamines.

The possibility also exists that the injections used in the present study included two functional subdivisions in each terminal area, and the resulting retrograde labeling in the two distinct areas of the SN-VTA is a reflection of this dual innervation. Our previous anatomical studies (Fallon and Moore, 1978b) demonstrated that nearly every telencephalic area having dopaminergic afferents receives inputs from both the SN and VTA, albeit from distinct dorsal-ventral domains, and that the projection from the VTA is to the medial segment of each nuclear area (e.g., caudate-putamen, nucleus accumbens, or olfactory tubercle) and the projection from the SN is to the lateral segment of the same nuclear areas. The recently discovered subpopulations of dopamine cells projecting to limbic areas, which also contain a cholecystokinin-like peptide (Hökfelt et al., 1980) overlap quite closely with the VTA cell projections to the medial, perhaps "limbic," component of each nuclear area. It is possible, therefore, that, in the present study, the injections included both the lateral "dopamine only" SN terminals and also the medial "dopamine plus cholecystokinin" VTA terminals. It may be that the cells containing the dopamine and cholecystokinin are present in the poorly collateralized neurons of the VTA.

It is still premature to conclude whether the differential degree of collateralization in the SN-VTA projections is a general phenomenon or just a circumstantial event related to the placement of injection sites. The injections were chosen because the terminal areas receive a moderately dense dopamine innervation arising from overlapping zones in the SN-VTA (Fallon and Moore, 1978b) and because they are anatomical and functional distinct areas of the telencephalon, that is, the striatal, limbic, and cortical systems. Thus, any sharing of input (collateralization) from a single mesencephalic cell would probably be a result of functional principles resident in the area of origin (SN-VTA) rather than the general topographical principle of projections operating in the same general terminal area, for example, the neostriatum.

It could be argued that the areas chosen were not functionally distinct but rather form a portion of the "prefrontal system" linking the prefrontal cortex and anteromedial striatum (Beckstead, 1979; Simon et al., 1979a; Divac and Diemer, 1980) or a cortical-subcortical loop linking the frontal cortex, septum, nucleus accumbens, and VTA (Thierry et al., 1979). It could also be argued that the systems chosen are independent. For example, the VTA cells projecting to the prefrontal cortex are functionally distinct from other VTA projections (Lisoprawski et al., 1980). Morphine has a differential effect on dopaminergic neurons projecting to the cortex and striatum (Moleman and Bruinvels, 1976), and the dorsal raphe regulates the activity of dopaminergic neurons projecting to nucleus accumbens but not those projecting to cortex (Simon et al., 1979b). Therefore, there are recently discovered (and classical) reasons to closely associate or widely dissociate the terminal areas chosen for study. The present demonstration of a new distinguishing characteristic in the SN-VTA neurons, that is, axonal collateralization, amplifies the connectional associations and dissociations of the striatal, limbic, and cortical systems by showing that one type of afferent can be subdivided into unique populations. One population, the ventral SN neurons, projects to many divergent telencephalic areas. The second population, exemplified by the VTA neurons, projects in a more one-to-one fashion to telencephalic areas. Does each cell in the complex neuropil of the VTA maintain a particular, perhaps singular, mosaic of afferent and efferent connections in a manner quite different from adjacent cells in the medial SN? We are presently investigating the collateralization of SN-VTA cells to other terminal areas in order to determine if the results of the present study represent a general feature of the projection of mesotelencephalic neurons.

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following injections illustrated in Figure 1. The *arrowhead* points to a cell labeled with GB, the *solid arrow* points to a cell labeled with NY, and the *open arrow* points to a cell labeled with PI. The black and white photograph was taken from an Ektachrome color slide exposure in 360- to 390-nm excitation light; therefore, the PI fluorescence (*open arrow*) is very faint. *D*, Photomicrograph taken from the *sn* showing the extensive double labeling of *sn* neurons after the injections illustrated in Figure 1. The *white arrows* point to two cells double-labeled with PI and GB, the *large arrows* point to two cells double-labeled with NY and GB, and the *open arrows* point to two cells labeled only with PI. In contrast to *C*, this photograph was taken from a black and white Pan-X exposure in 360- to 390-nm excitation light. Again, the PI fluorescence (*open arrows*) is faint. Full appreciation of the fluorescence labeling can be demonstrated only with color photomicrographs.

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