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Organization of the Ascending Projections From the Ventral Tegmental Area: A Multiple Fluorescent Retrograde Tracer Study in the Rat

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ABSTRACT

The projections from the ventral tegmental area of Tsai (VTA) to the frontal cortex (FC), lateral septum (LS), nucleus accumbens (Acc), caudate-putamen (CPu), and lateral habenula (LH) were investigated in the rat by means of the double retrograde fluorescent tracer technique.

True blue and fast blue were used in combination with nuclear yellow as retrograde tracers. After combined injections placed into two different terminal fields, many singly and some doubly labeled neurons were seen in the midbrain. In all cases the labeled cells were observed in the ipsilateral VTA, while after injections placed into the LS and Acc some fluorescent neurons were also seen in the contralateral VTA. The patterns of distribution of the labeled neurons showed a topographic organization of the VTA efferent pathways. However, some degree of overlapping was evident in the distribution of cells retrogradely labeled from different terminal fields. The number of the doubly labeled neurons varied according to the sites of combined injections, but in each experiment it never exceeded 10% of the total number of labeled perikarya. Doubly labeled neurons were particularly numerous after combined injections placed into the FC, LS, or LH; on the contrary, very few doubly labeled cells were observed after combined injections placed into the CPu and LS or LH.

The organization of the ascending VTA projections suggests that they are probably integrated into different anatomical sets.

Key words: frontal cortex, lateral septum, nucleus accumbens, lateral habenula, limbic system, dopamine pathways

The ascending projections of the ventral tegmental area (VTA) are distributed over several regions of the forebrain, namely, the frontal, suprarhinal, and entorhinal cortices, the amygdala, the lateral septum, the olfactory tubercle, the striatum, the nucleus accumbens, the hippocampus, and the lateral habenular nucleus (Herkenham and Nauta, '77; Fallon and Moore, '78; Nauta et al., '78; Beckstead et al., '79; Simon et al., '79; Phillipson and Griffith, '80; Scatton et al., '80). Such an array of projections to regions belonging to different anatomical circuitries accounts for uncertainty in attributing a definite functional significance to the VTA. However, from many studies it seems that the VTA plays a role in the control of simple motor activity and complex behavior (Le Moal et al., '75; Fink and Smith, '80; Javoy-Agid and Agid, '80); this action is probably exerted via different sets of anatomical connections, such as the ascending projections to regions belonging to the extrapyramidal system or to the limbic system.

The VTA contains the dopaminergic cell bodies of the A10 cell group (Dahlström and Fuxe, '64) and histofluorescence studies have described dopaminergic efferent projections from the VTA (see reviews in Lindvall and Björklund, '78; Moore and Bloom, '78). Immunohistochemical studies have also shown the presence of cholecystokinin- and enkephalin-containing neurons in the VTA (Hökfelt et al., '80; Johnson et al., '80). Furthermore, it is known from morphological studies (Phillipson, '79a) that the VTA can be subdivided into cytoarchitectonically distinct cell groups, which all contain dopaminergic neurons and possibly give rise to different efferent pathways. Catechol-

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amine-containing neurons are known to have a rich collateralization pattern (Lindvall and Björklund, '74), and it has also been shown that axons arising from neurons located in the VTA give off recurrent collaterals to their parent cell bodies (Phillipson, '79b). Taken together, all these data suggest that the anatomical organization of the VTA cells as well as of their efferent pathways is rather complex and diversified.

Therefore, the question arose as to whether the VTA efferent fibers, which are distributed to limbic and striatal regions of the forebrain, in fact represent collaterals of the same ascending axons. This problem has been investigated only in part, both electrophysiologically by the antidromic activation technique (Deniau et al., '80) and anatomically by means of fluorescent tracers (Fallon, '81). In order further to define the hodological organization of some functionally significant circuitries originating in the ventral midbrain tegmentum, the double fluorescent retrograde tracer technique (Kuypers et al., '80) was used in the present study to trace the VTA efferent connections to the frontal cortex (FC), lateral septal nucleus (LS), caudate-putamen (CPu), nucleus accumbens (Acc), and lateral habenular nucleus (LH). The present report describes the detailed topography of mesencephalic neurons projecting to these regions and shows the presence of cell bodies giving off divergent axon collaterals to different target territories.

MATERIALS AND METHODS

The present study is based on a total of 39 male Wistar rats, weighing 250–300 gm. The animals were deeply anesthetized with sodium pentobarbital (40 mg/kg) before undergoing surgery.

Three fluorescent retrograde tracers were used (Bentivoglio et al., '79a, '80b): true blue (TB, 2% aqueous suspension), fast blue (FB, 2% aqueous suspension), and nuclear yellow (NY, 1% aqueous suspension). Under appropriate light conditions, TB and FB fluoresce blue and label chiefly the neural cytoplasm: They were alternatively injected in combination with NY which, under the same conditions, fluoresces yellow and labels chiefly the neural nucleus. Each animal received one injection of either TB or FB and one of NY in two different regions of the same cerebral hemisphere, by means of two different Hamilton microdrive microsyringes. With the exception of the cortical injections, all needle insertions were placed stereotaxically, according to the atlas by Pellegrino and Cushman ('67).

The experiments can be subdivided in seven groups (see Table 1): *Group A* consisted of four animals in which one fluorophore was injected into the FC and the other into the LS; *group B* consisted of six animals in which the injections were placed into the FC and into the LH; *group C* consisted of six animals in which the injections were made into the CPu and into the LS; *group D* consisted of five animals in which the injections were made into the CPu and the LH; *group E* consisted of six animals in which one tracer was injected into the Acc and the other into the LS; *group F* consisted of five animals in which the injections were made into the LS and the LH; *group G* consisted of seven animals in which the injections were placed into the Acc and into the LH. Following an injection, the syringe was left in place for 10 minutes before removal from the brain: This procedure greatly limits the spillage of tracers along the needle tracks. In order to prevent the migration of NY out of retrogradely labeled neurons, which can be detected by fluorescent labeling of the adjacent glial nuclei (Bentivoglio et al., '80a), survival after the NY injections varied from 18 to 22 hours (Table 1). Since survival after TB injections varied from 4 to 8 days, while after the FB injections it was 3–5 days (see Table 1), two different surgical sessions, one for the TB or FB injections and a second for the NY injection, were performed in each experiment.

All the animals were perfused transcardially with saline followed by 10% buffered formalin (pH 7.2). The brains were soaked in 30% buffered sucrose (pH 7.2) for a period ranging from 4 to 10 hours, then cut on a freezing microtome into 30-μm-thick sections, which were immediately mounted on slides from distilled water and air dried. The sections were studied, without coverslipping, under a Leitz Ploemopak fluorescence microscope using a 340–380-nm light excitation wavelength and a 430-nm barrier filter (filter-mirror system A). Serial sections through the injection sites were charted for the reconstruction of the size of the injection area; the distribution of labeled neurons was charted on serial drawings of the ventral midbrain tegmentum. In each case, the singly and doubly labeled perikaria were counted while charting, and the number of doubly labeled neurons was indicated as percentage of the total population of labeled cells. In order to avoid decrease of fluorescence, which may occur during the exposure of tissue specimens to the ultraviolet light, the photographic documentation was taken either after charting or on different sections from those chosen for the charts. The charts were compared with Nissl-stained standard series of the rat brain and were additionally checked by counterstaining some sections with cresyl violet after charting.

RESULTS

Delimitation of ventral tegmental area

In order to draw guidelines for the description of VTA-labeled neurons, it is convenient to briefly outline the position and boundaries of VTA. Tsai ('25) first described it as *nucleus ventralis tegmentalis*; but, due to its loose neuronal texture, the term *area*, rather than *nucleus*, has entered common terminology. The VTA is located in the mesencephalon, medial to the cerebral peduncle and to the medial lemniscus, and ventral to the red nucleus; ventrally, the area directly abuts the interpeduncular nucleus. The VTA has ill-defined boundaries; rostrally and caudally it merges with the diencephalic and mesencephalic reticular formations, respectively; laterally it is continuous with the pars compacta of the substantia nigra (SNC) and with the supralemniscal area. Cytoarchitectonic studies in the cat and rat have shown that the ventral midbrain tegmentum contains three identifiable cell groupings: the nucleus paranigralis, the nucleus parabrachialis pigmentosus, and the interfascicular nucleus. In the rat brain, these nuclei are contained within the boundaries of the VTA (Taber, '61; Phillipson, '79a). As demonstrated with histofluorescence studies, the VTA is composed largely of dopamine neurons (cell group A10), and also contains nondopaminergic neurons, which are intermixed with the catecholamine-containing perikarya (van der Kooy et al., '81; Albanese, '82; Albanese and Bentivoglio, '82). Therefore, it is convenient to consider the rostrocaudal extent of the A10 dopamine cell group as a reliable criterion to define the rostral and caudal boundaries of the VTA. In the light of this consideration, and in accord with two recent topographic histofluorescence studies (Fallon and Moore, '78; Phillipson,

TABLE 1. Synopsis of Experimental Data

Group	Case no.	Injection site[1]	Tracer (μl)[2]	Survival (days)	Injection site[1]	Tracer (μl)[2]	Survival (hours)
A	D 140	LS	TB (0.15)	6	FC	NY (1.0)	20
	D 144	LS	TB (0.15)	5	FC	NY (1.0)	22
	D 183	LS	TB (0.2)	4	FC	NY (1.2)	18
	D 187	LS	TB (0.1)	6	FC	NY (0.6)	20
B	D 162	LH	FB (0.025)	5	FC	NY (1.0)	22
	D 172	LH	TB (0.03)	7	FC	NY (1.0)	18
	D 173	LH	TB (0.025)	7	FC	NY (1.2)	18
	D 181	LH	TB (0.015)	4	FC	NY (0.75)	20
	D 192	LH	TB (0.03)	6	FC	NY (1.2)	20
	D 203	LH	FB (0.025)	3	FC	NY (0.7)	20
C	D 138	CPu	TB (0.6)	6	LS	NY (0.3)	18
	D 139	CPu	TB (0.4)	6	LS	NY (0.2)	20
	D 175	LS	TB (0.075)	7	CPu	NY (0.2)	17
	D 193	LS	TB (0.15)	6	CPu	NY (0.2)	18
	D 200	LS	FB (0.15)	4	CPu	NY (0.3)	18
	D 201	LS	FB (0.1)	4	CPu	NY (0.2)	18
D	D 163	LH	FB (0.025)	4	CPu	NY (0.2)	18
	D 174	LH	TB (0.025)	8	CPu	NY (0.2)	16
	D 188	LH	FB (0.025)	5	CPu	NY (0.15)	20
	D 190	LH	TB (0.025)	8	CPu	NY (0.15)	18
	D 198	LH	FB (0.025)	3	CPu	NY (0.2)	18
E	D 152	LS	TB (0.1)	6	Acc	NY (0.15)	20
	D 153	Acc	TB (0.1)	5	LS	NY (0.15)	20
	D 184	LS	TB (0.15)	4	Acc	NY (0.2)	18
	D 185	LS	TB (0.1)	8	Acc	NY (0.1)	18
	D 209	LS	TB (0.1)	4	Acc	NY (0.1)	17
	D 210	LS	TB (0.1)	4	Acc	NY (0.1)	17
F	D 161	LH	FB (0.05)	3	LS	NY (0.1)	20
	D 165	LH	FB (0.025)	3	LS	NY (0.1)	18
	D 166	LH	FB (0.025)	3	LS	NY (0.1)	18
	D 195	LH	FB (0.025)	3	LS	NY (0.15)	20
	D 196	LH	FB (0.025)	4	LS	NY (0.2)	19
G	D 160	LH	FB (0.025)	4	Acc	NY (0.2)	18
	D 176	LH	TB (0.025)	6	Acc	NY (0.15)	21
	D 182	LH	TB (0.025)	4	Acc	NY (0.2)	16
	D 186	LH	TB (0.025)	8	Acc	NY (0.1)	18
	D 189	LH	TB (0.025)	4	Acc	NY (0.1)	19
	D 191	LH	TB (0.025)	8	Acc	NY (0.1)	18
	D 204	LH	FB (0.025)	3	Acc	NY (0.15)	19

[1]LS, lateral septum; LH, lateral habenula; CPu, caudate-putamen; Acc, nucleus accumbens; FC, frontal cortex.
[2]TB, true blue; FB, fast blue; NY, nuclear yellow.

'79a), we assume here as the rostralmost level of VTA a section just rostral to the medial accessory optic nucleus and crossing the mammillary body and the tegmental decussatio; we assume the caudalmost level is a section just caudal to the tegmental decussatio and to the red nucleus.

Injection sites

The TB injection areas showed two concentric zones around the needle track (Huisman et al., '81). In the cases injected into the LS, the first zone involved only the LS, while the second zone extended medially into the ipsilateral medial septum and laterally up to the ependyma of the lateral ventricle. In order to avoid crossing the FC, in the experiments of group A the septal injections were performed with the syringe sloped at a 45° angle from rear to front. In the cases injected into the LH the first zone involved most of the LH and the stria medullaris, while the second zone was very narrow and did not extend beyond the borders of the LH. In cases D138 and D139, in which the CPu was injected, the overall injection site was quite big; the second zone extended dorsally and laterally up to the corpus callosum, medially to the border of the lateral ventricle, and ventrally it did not reach the anterior commissure. In case D153, in which the Acc was injected, the first zone was just medial to the anterior commissure and the second zone did not exceed the boundaries of the nucleus.

In the charts of FB injection sites two concentric zones were outlined: The central zone corresponded to the first and second zone depicted by Bharos et al. ('81), while the peripheral zone corresponded to the third zone of the same authors. In the cases injected into the LS the two zones were quite similar to those observed with the injection of TB. In the cases injected into the LH, the central zone was always restricted to this nucleus; the peripheral zone ventrally did not exceed the fasciculus retroflexus, and in some cases it extended medially into the medial habenula. The injection site of case D204 is shown in Figure 1.

The NY injection sites were also divided into two concentric zones: The central zone corresponded to the first and second zone described by Bharos et al. ('81) and by Huisman et al. ('81); the peripheral zone corresponded to the third zone of the same authors. In the cortical injections, the two zones involved large portions of the frontal pole and of the supragenual and the anterior cingulate cortices. In the cases injected into the CPu, the injection sites involved different parts of this nucleus; in some cases (D190, D198, D200, and D201) the NY injections involved the ros-

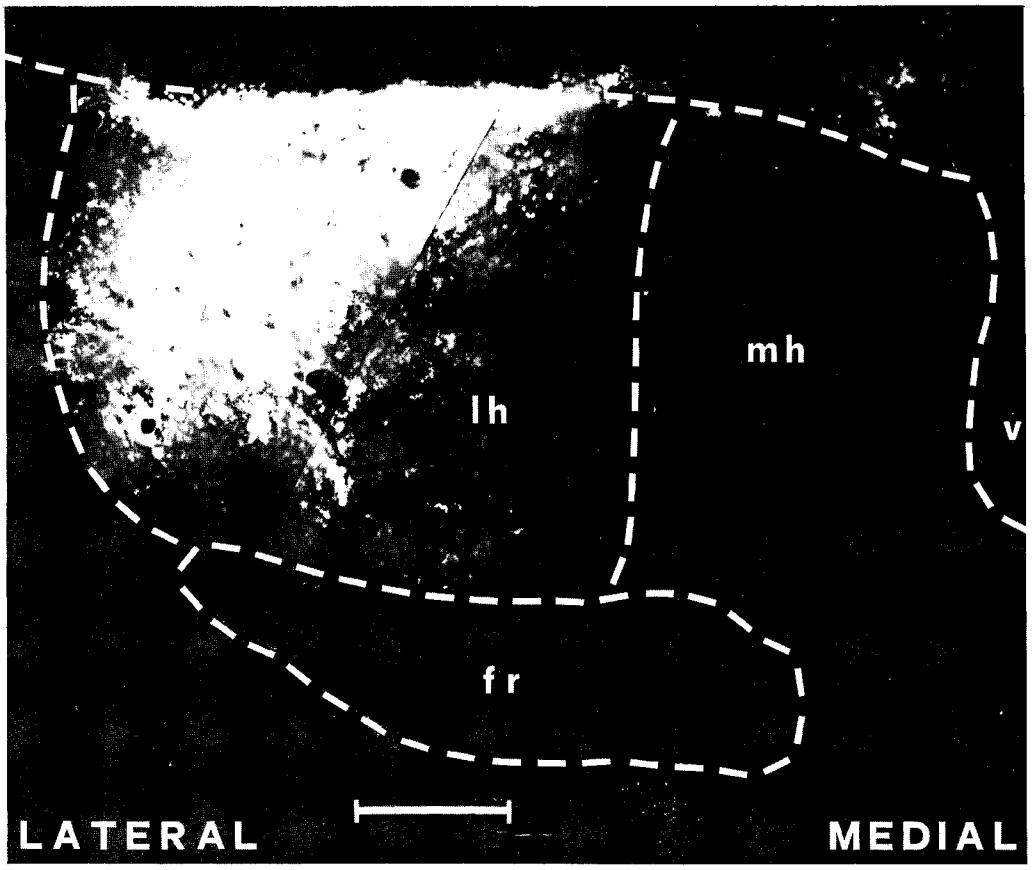

Fig. 1. Microphotomontage of a fast blue (FB) injection into the LH (case D204). Coronal section; scale bar = 250 μm.

tral part of CPu, i.e., at the same rostrocaudal level of the Acc, while in the other cases the injection areas were located more caudally. However, the NY injections never involved the entire volume of the CPu. In the case injected into the LS, the peripheral zone extended medially to the ipsilateral medial septum, while laterally it did not exceed the border of the lateral ventricle. In the cases injected into the Acc the two zones involved the anterior commissure and this nucleus, with the exception of two cases (D160, D209) in which the peripheral zone extended medially far enough to involve the nucleus of the diagonal band.

Topography of retrogradely labeled neurons

The distribution of flourescent retrogradely labeled neurons in the mesencephalon varied according to the topography of the injection sites.

After injections placed into the FC the retrogradely labeled neurons were found ipsilaterally in the VTA and in the SNC. At rostral levels, the fluorescent cells were predominantly located in the medial third of the SNC and in the adjacent ventral part of the VTA, which corresponds to the nucleus paranigralis; at more caudal levels labeled

Abbreviations

Acc	Nucleus accumbens	Mh	Medial habenula
Aon	Medial accessory optic nucleus	Ml	Medial lemniscus
Cp	Cerebral peduncle	Mp	Mammillary peduncle
Cpu	Caudate-putamen	Pmb	Posterior mammillary body
Fr	Fasciculus retroflexus	R	Red nucleus
Hpc	Hippocampus	Snc	Substantia nigra pars compacta
Ip	Interpeduncular nucleus	Snr	Substantia nigra pars reticulata
Lh	Lateral habenula	V	Third ventricle

neurons were also found in the medial VTA, near the border of the interpeduncular nucleus. With the exception of a few neurons situated just beyond the midline, no labeled neurons were seen in the contralateral mesencephalon.

In the cases injected into the LS many retrogradely labeled cells were found in the ipsilateral VTA; they were located medially, close to the border of the interpeduncular nucleus, and also in the midline, in the interfascicular nucleus and within the fibers of the tegmental decussatio; some labeled cells were always found more ventrally, proximate to the fibers of the mammillary peduncle. In addition, some retrogradely labeled neurons were located in the medial part of the contralateral VTA.

Injections placed into the CPu caused retrograde labeling of the ipsilateral SNC and VTA. Particularly the SNC was filled with labeled neurons; the labeled cells in the VTA were prevalently located in the lateral part, close to the medial lemniscus, or medially, near or just beyond the midline. In fact, retrograde labeling of the contralateral VTA consisted of a small number of fluorescent cells lying not far from the midline and probably representing displaced ipsilateral perikarya.

In the cases injected into the Acc, retrogradely labeled neurons were present in the medial portion of the SNC, but they were particularly numerous in the VTA. In this area, the labeled cells were predominantly distributed in the midline: At rostral levels they were located between the two fasciculi retroflexi and in the interfascicular nucleus, while more caudally they were distributed above the interpeduncular nucleus. More laterally in the VTA, many fluorescent cells were seen around the medial lemniscus and within the rootlets of the oculomotor nerve. Neurons labeled from Acc injections were rarely found in the ventral portion of the VTA; this was especially true in those cases in which the Acc injections were rather small (see Fig. 8). However, some fluorescent neurons were always found in the contralateral side; they were located somewhat more laterally than those labeled from septal injections.

In the cases injected into the LH the retrogradely labeled cells were predominantly fond in the dorsal part of the ipsilateral VTA; they were more numerous at rostral levels, particularly in a region situated between the medial lemniscus and the fasciculus retroflexus. No labeled cells were found contralaterally.

Collateralization of VTA efferent pathways

In all the experiments of combined injections, the retrogradely doubly labeled neurons represented a minority of the total number of retrogradely labeled cells. The doubly labeled neurons were always located only ipsilaterally to the sites of injections. In agreement with previous observations (De Olmos and Heimer, '80), it was noted that the doubly labeled cells were not as brilliantly fluorescent as the singly labeled ones; notwithstanding, the identification of doubly labeled neurons was never doubtful.

After combined injections placed into the FC and LS (group A), the doubly labeled cells varied from 5 to 10% of the total number of labeled perikarya and were predominantly distributed in the dorsolateral part of the ipsilateral VTA (Fig. 3). After combined injections placed into the FC and LH (group B), the doubly labeled neurons varied from 5 to 10% of the total; they were more numerous at rostral levels in the dorsolateral part of the VTA (Fig. 4). After combined injections placed into the CPu and LS (group C), very few retrogradely doubly labeled neurons were seen in the VTA; their number varied from 1 to 2% of the total number of labeled cells (Fig. 5). After combined injections placed into the CPu and LH (group D), the doubly labeled neurons were 2–3% of the total; in rostral sections of the mesencephalon these cells were located in the dorsal part of the VTA. After combined injections into the Acc and LS (group E), the doubly labeled neurons were found in the ipsilateral VTA, where they represented 2–5% of the total number of labeled cells (Fig. 6). In the cases injected into the LS and LH (group F), the retrogradely doubly labeled neurons were distributed in the dorsolateral and rostral portion of the ipsilateral VTA; they varied from 7 to 10% of the total population of labeled perikarya (Fig. 7). Finally, in the cases injected into the Acc and LH (group G), the doubly labeled neurons were found in the rostral part of the VTA, where they represented 3–5% of the total population (Fig. 8).

DISCUSSION

The present data show that mesencephalic neurons projecting to the forebrain display a definite topographic organization; thus, the distribution of neurons retrogradely labeled after injections placed into the FC, LS, CPu, Acc, and LH is consistently reproducible in different experiments. However, as this study shows, VTA neurons projecting to different target territories are not clustered into subnuclei but are instead partially intermixed.

The population of neurons giving off divergent axon collaterals does not exceed, in each experiment, 10% of the total population of retrogradely labeled cells. However, the number of labeled perikarya varied in the different groups of combined injections, thus suggesting an organized pattern of collateralization. In fact, very few doubly labeled neurons could be detected in the mesencephalon after combined injections involving limbic and striatal areas (e.g., groups C, D); on the contrary, the number of doubly labeled cells tended to be higher after combined injections placed into two limbic structures (e.g., groups A, B, F). Therefore, on the basis of the double-labeling experiments it is apparent that the *limbic* and *striatal* populations of VTA neurons exchange little communication through direct axonal arborization. However, the present study does not deal with two of the known target territories of the VTA ascending system, the amygdala and the entorhinal cortex, which are both believed to play an important role in the limbic system. This problem is an object for future studies, which will probably make it possible to complete the findings reported here.

The present study also shows that mesencephaloaccumbens neurons have a somewhat different organization from the rest of VTA rostrally projecting neurons. After combined injections involving the Acc and the LS or LH (groups E and G), the number of retrogradely double-labeled neurons was approximately 3–5% of the total population, i.e., higher than the number of cells labeled from CPu and LS or LH combined injections (groups C and D). Second, mesencephaloaccumbens neurons differ from the rest of VTA cells in that they are distributed throughout the ipsilateral ventral tegmentum; this finding is in keeping with the retrograde study by Nauta et al. ('78) and does not support the data by Fallon and Moore ('78) concerning a medioventral clustering of the mesencephaloaccumbens cells. Furthermore, in agreement with previous studies in

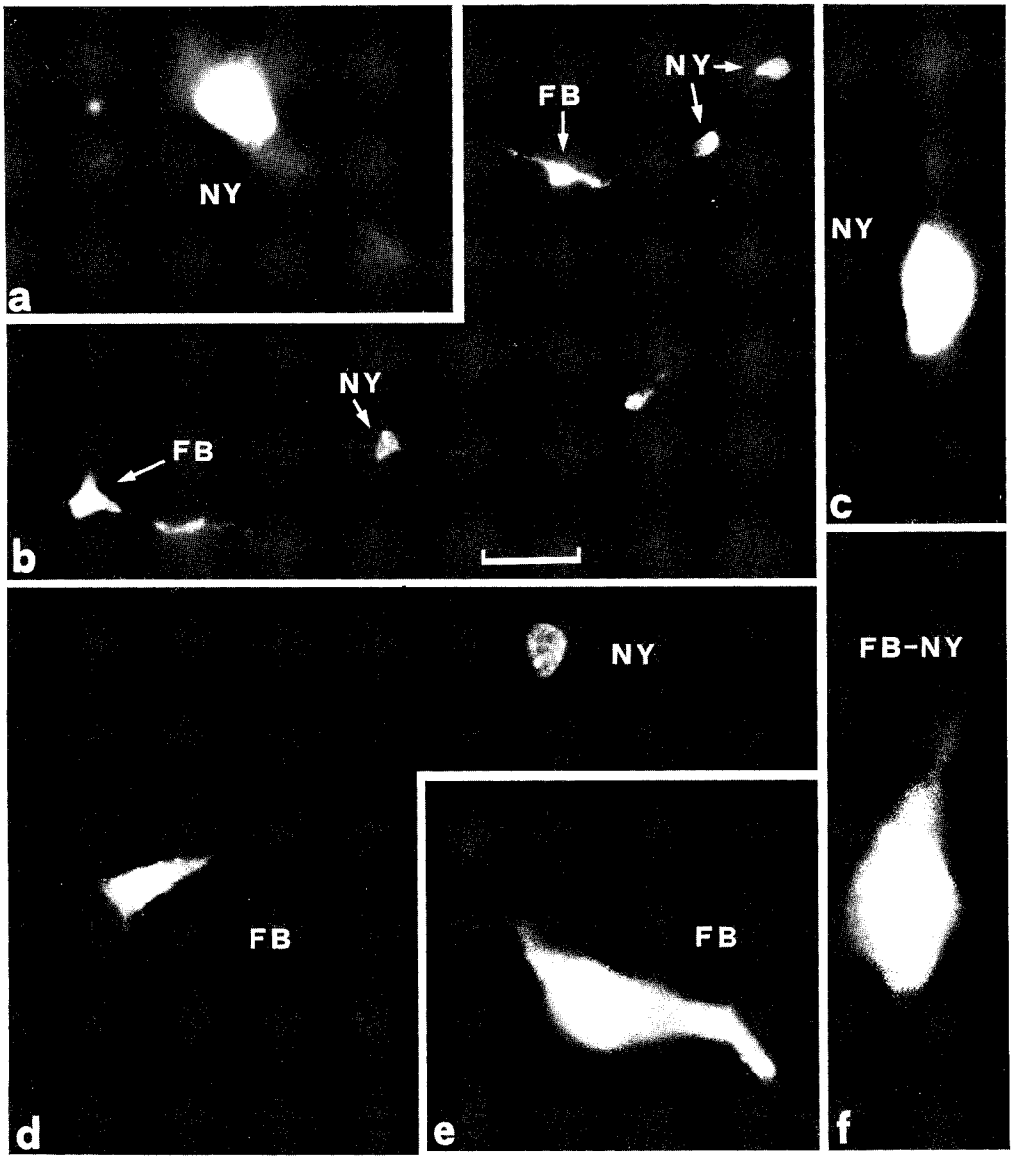

Fig. 2. Microphotographs of fluorescent retrogradely labeled ventral tegmental area (VTA) neurons. a,c. Nuclear yellow (NY)-labeled neurons with brilliantly fluorescent nuclei and some NY cytoplasmatic fluorescence. b. NY and FB singly labeled neurons located in the rostral part of VTA (case D200). d. NY and FB singly labeled neurons located in the VTA (case D201). e. FB-labeled neuron with blue fluorescent FB-labeled cytoplasm and proximal dendrites. f. FB-NY doubly labeled neuron displaying a yellow fluorescent NY-labeled nucleus and blue fluorescent FB-labeled cytoplasm and proximal dendrites. Scale bar: for a, c, e, f = 12 μm; for b = 7.5 μm; for d = 6 μm.

the rat (Chronister et al., '80) and in the cat (Groenenwegen et al., '80), our data demonstrate that the mesencephaloaccumbens pathway is bilateral in origin. Anterograde tracing studies (Beckstead et al., '79; Simon et al., '79) have shown that this pathway represents the major efferent system of the VTA. Morphologically, the Acc belongs to the striatum, but many studies have pointed out that this nucleus is functionally related to the limbic system (see Nauta et al., '78; Johansson and Hökfelt, '81). Mogenson et al. ('80) have also suggested that the Acc may constitute a transitional zone or a link between the basal ganglia and the limbic system. Since a similar role has been attributed to the VTA, it is likely that the mesencephaloaccumbens pathway plays a key role in connecting striatal and limbic circuitries.

The distribution of mesencephalocortical, mesencephaloseptal, mesencephalostriatal, and mesencephalohabenular neurons, as shown in the present report, is in keeping with previous anterograde and retrograde studies (Beckstead, '76; Carter and Fibiger, '77; Herkenham and Nauta, '77; Björklund and Lindvall, '78; Fallon and Moore, '78; Nauta et al., '78; Beckstead et al., '79; Bentivoglio et al., '78, '79b; Simon et al., '79; Phillipson and Griffith, '80; Fallon, '81; Albanese and Bentivoglio, '82). However, the present study adds to existing descriptions some new details that may be relevant to our knowledge of the organization

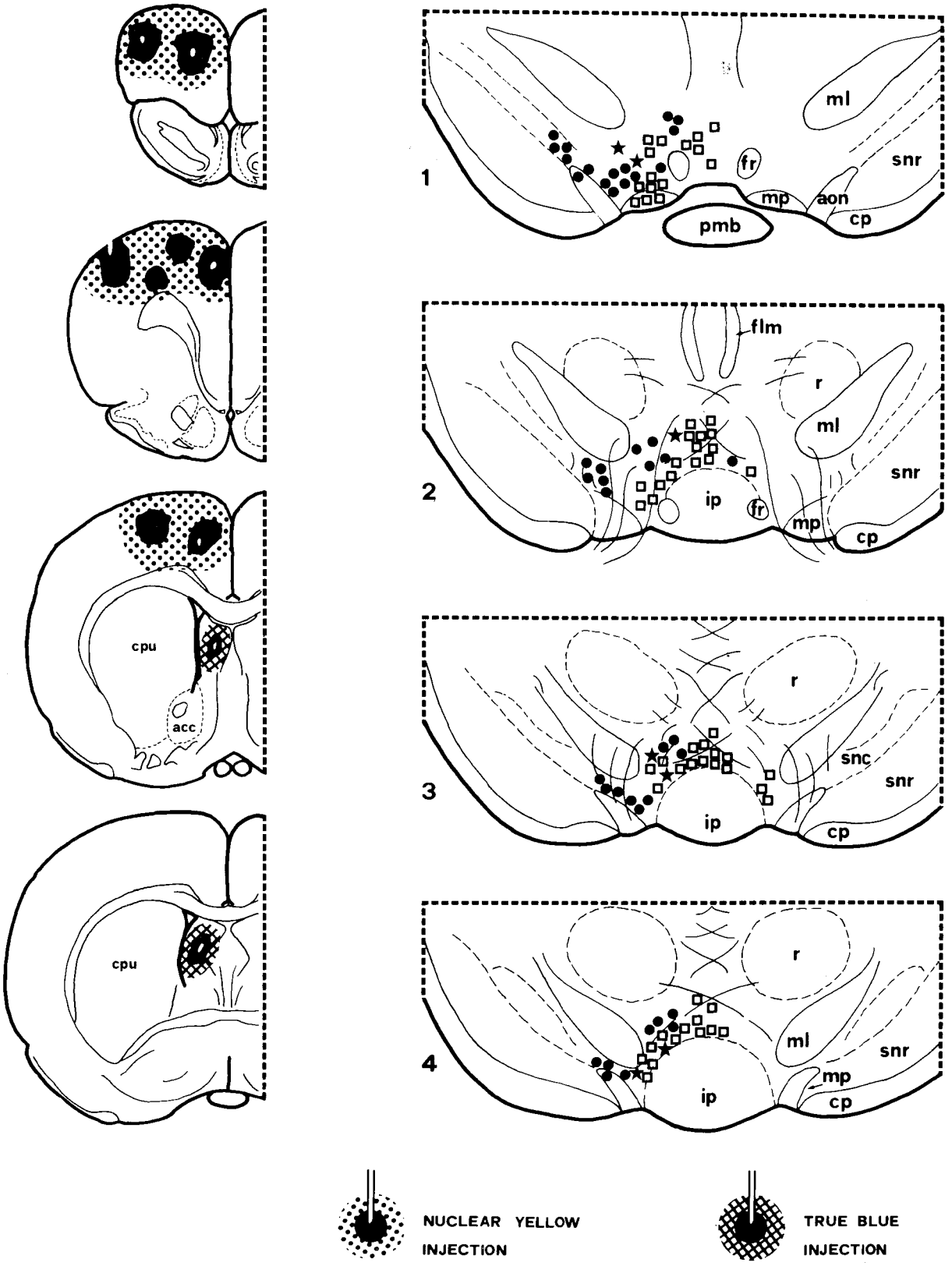

Fig. 3. Schematic representation of the extent of the true blue (TB) and NY injection areas in case D183 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each black dot corresponds to two NY singly labeled cells, each open square represents two TB singly labeled cells, and each star corresponds to two TB-NY doubly labeled neurons. Numbers 1–4 indicate the rostrocaudal transverse sections.

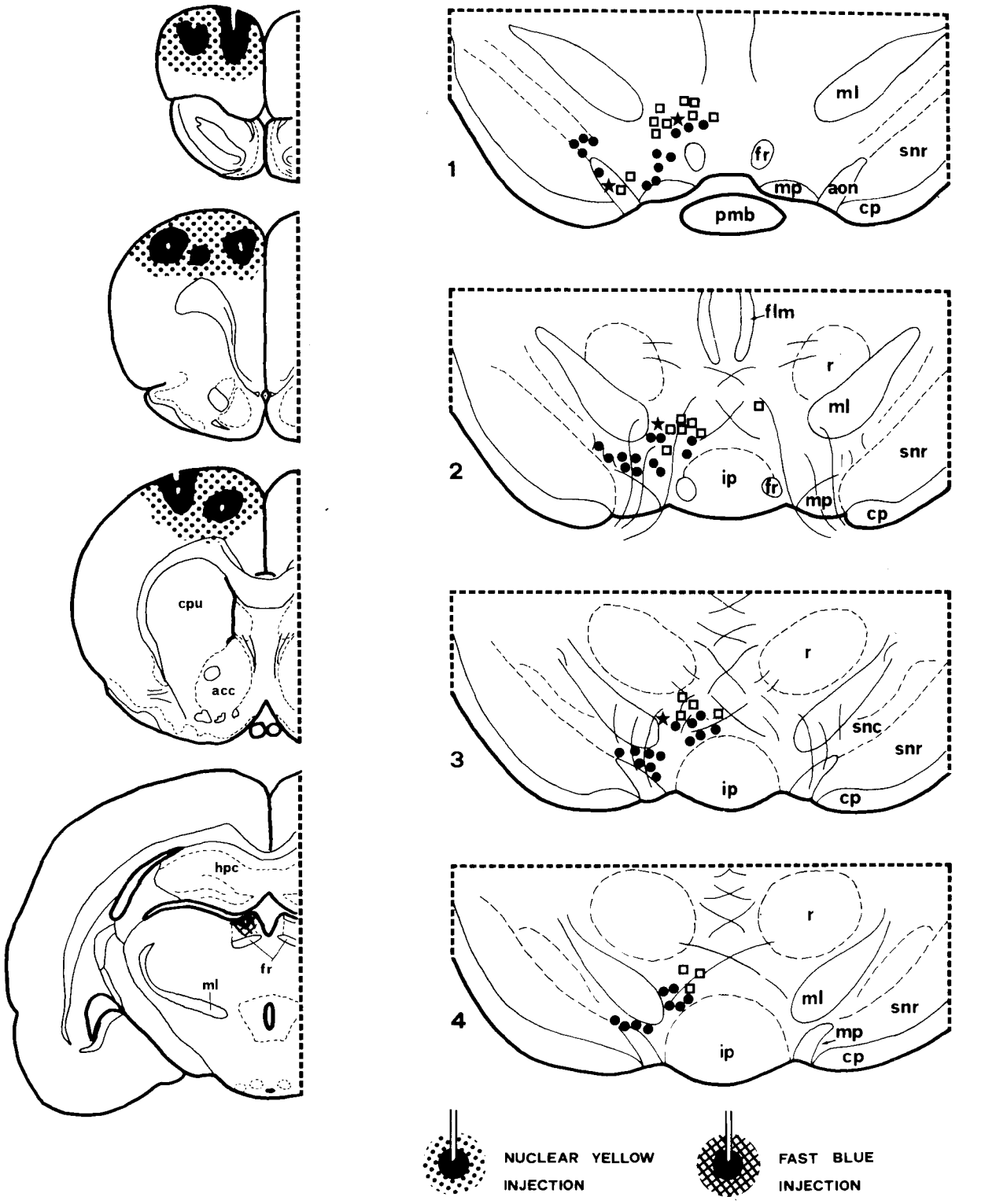

Fig. 4. Schematic representation of the extent of the FB and NY injection areas in case D162 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each black dot corresponds to two NY singly labeled cells, each open square corresponds to two FB singly labeled cells, and each star corresponds to two FB-NY doubly labeled neurons. Numbers 1–4 indicate the rostrocaudal transverse sections.

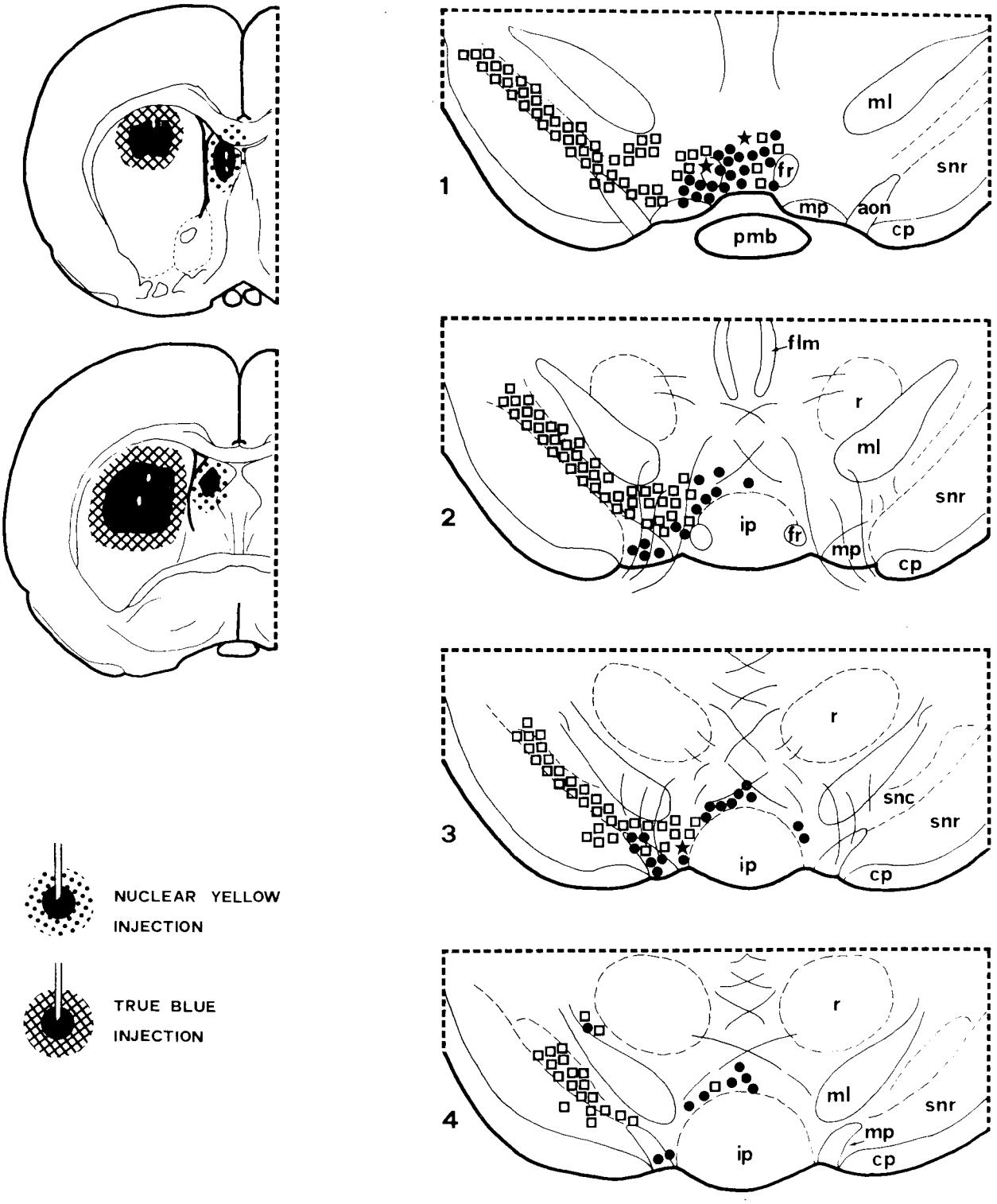

Fig. 5. Schematic representation of the extent of the TB and NY injection areas in case D139 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum, in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each dot corresponds to two NY singly labeled cells, each open square in the Snc corresponds to three TB singly labeled cells, each open square outside the Snc corresponds to two TB singly labeled neurons, and each star corresponds to one TB-NY doubly labeled cell. Numbers 1-4 indicate the rostrocaudal transverse sections.

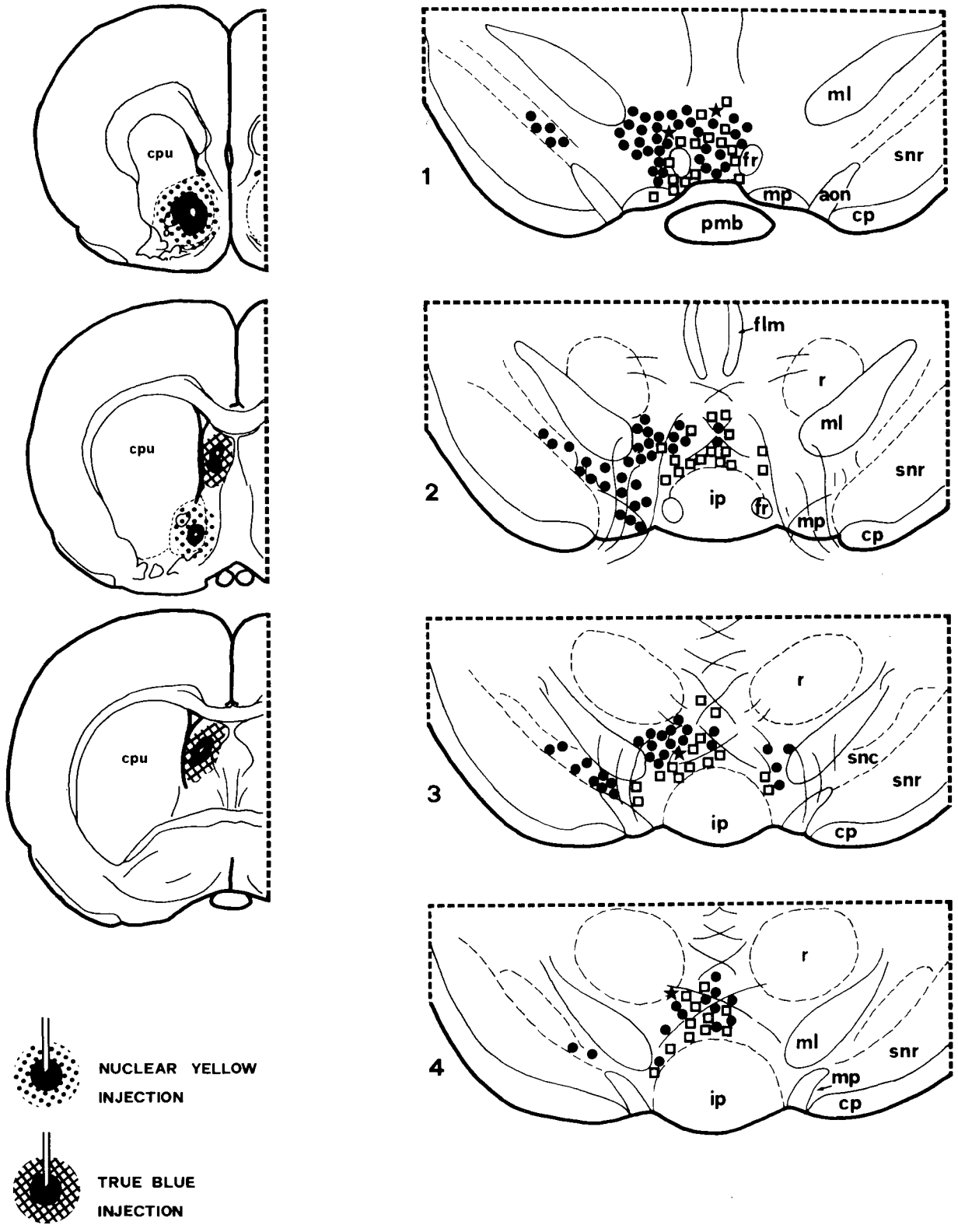

Fig. 6. Schematic representation of the extent of the TB and NY injection areas in case D209 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each dot corresponds to two NY singly labeled neurons, each open square corresponds to two TB singly labeled neurons, and each star corresponds to two TB-NY doubly labeled neurons. Numbers 1–4 indicate the rostrocaudal transverse sections.

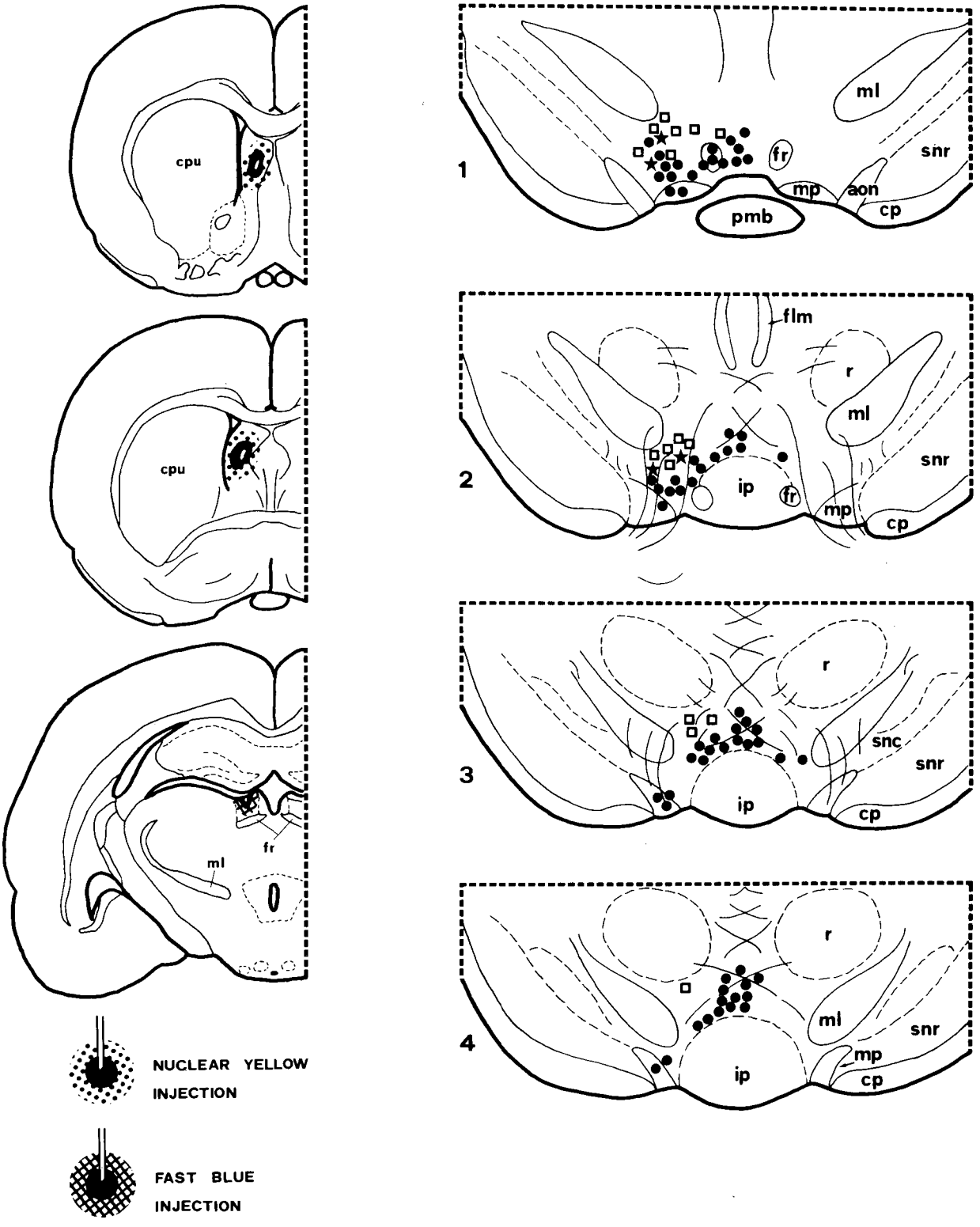

Fig. 7. Schematic representation of the extent of the FB and NY injection areas in case D195 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each dot corresponds to two NY singly labeled cells, each open square corresponds to two FB singly labeled cells, and each star corresponds to two FB-NY doubly labeled neurons. Numbers 1-4 indicate the rostrocaudal transverse sections.

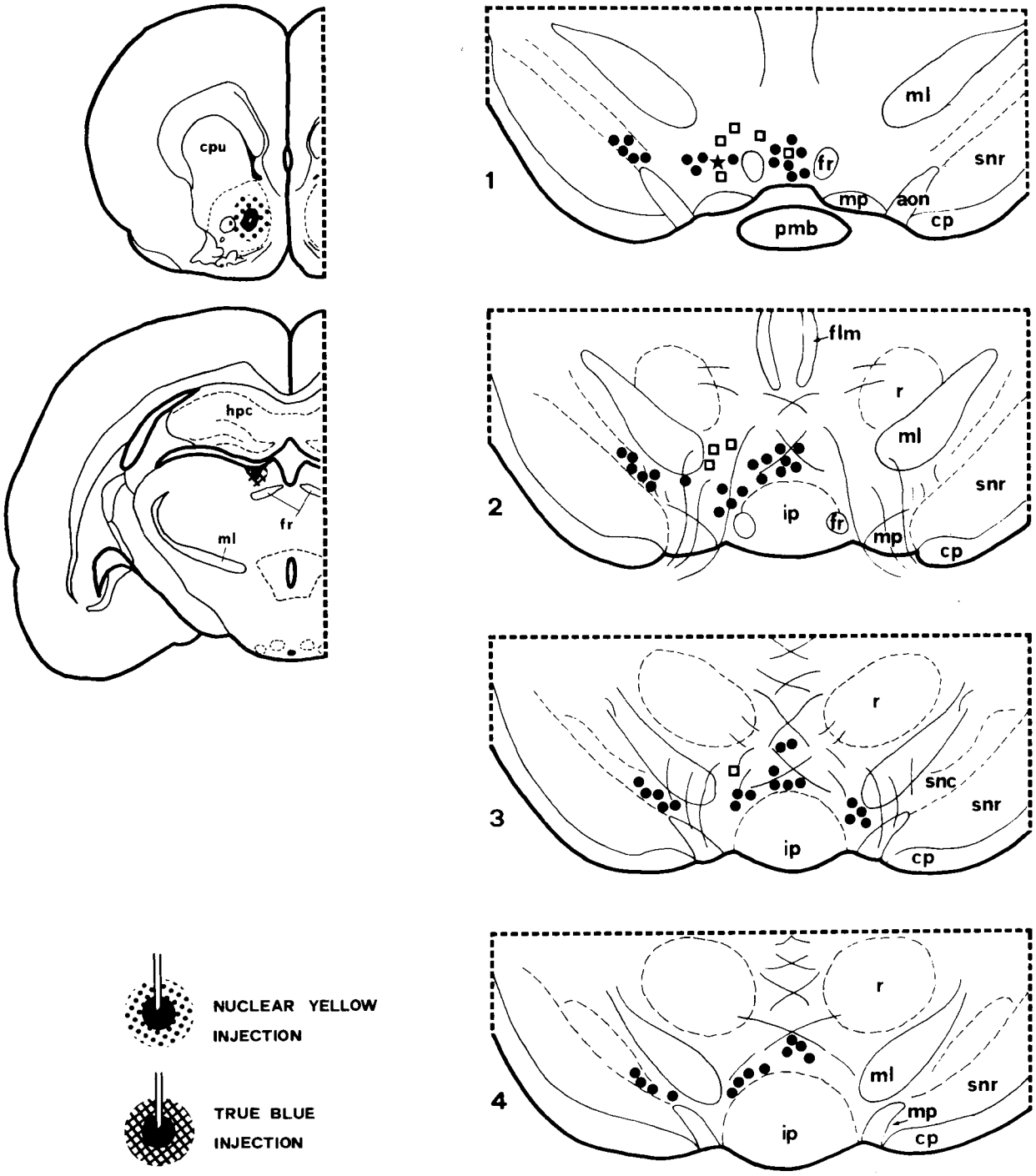

Fig. 8. Schematic representation of the extent of the TB and NY injection areas in case D191 (left column) and of the distribution of singly and doubly labeled cells in the ventral midbrain tegmentum in the same case (right column). The central and peripheral zones of the injection areas (see text) have been drawn schematically. Each dot corresponds to two NY singly labeled cells, each open square corresponds to two FB singly labeled cells, and each star corresponds to two FB-NY doubly labeled neurons. Numbers 1-4 indicate the rostrocaudal transverse sections.

of the ascending VTA efferent pathways. For the first time the presence of a crossed mesencephaloseptal projection originating from the VTA is reported. After HRP injections into the LS, which also extended into the medial septum, Fallon and Moore ('78) did not find retrogradely labeled perikarya in the contralateral VTA. Differences in sensitivity between the standard HRP technique and the fluorescent retrograde tracing method may account for this discrepancy.

The topographic distribution of VTA neurons giving rise to divergent axon collaterals is reported here for the first time. The present results show that the doubly labeled VTA neurons are distributed ipsilaterally to the injection sites. This is also true in the case of combined LS and Acc injections (group E), where single TB and NY, but no TB-NY doubly labeled cells were found in the contralateral VTA. These data are in keeping with the electrophysiological study by Deniau et al. ('80), and with the anatomical report by Fallon ('81), who describes the occurrence of doubly and triply labeled neurons in the VTA after injections placed into the FC, Acc, and LS.

The use of the multiple fluorescent retrograde tracer technique is one of the few anatomical methods suitable for demonstrating the existence of divergent axon collaterals in neural pathways. This technique appears to be more sensitive than other available multiple or single retrograde tracing methods (Huisman et al., '82); however, there is still the possibility that the fluorescent retrogradely double-labeled neurons underestimate the actual population of neurons possessing long axon collateals (De Olmos and Heimer, '80). This aspect certainly requires further analysis. The multiple fluorescent retrograde tracer technique, however, has hitherto been used in the study of many ascending and descending systems in the brain (Bentivoglio et al., '79b; Bharos et al., '81; Huisman et al., '81). Its application to the ascending pathways of the VTA presents some difficulties especially in the rat, due to the close proximity of some of the injected structures to the fiber bundles ascending from the brainstem. This problem is crucial in the case of injections placed into the Acc, which lies very close to the medial forebrain bundle, a major route containing ascending pathways bound to the FC and CPu, and also in the case of injections placed into the CPu, which necessarily involve the fibers crossing this nucleus. In the light of these considerations, the present study does not deal with combined injections involving the Acc and the FC or the Acc and the CPu, which would have led to dubious retrograde double labeling in the VTA. Second, it is also possible that neurons singly labeled from Acc or CPu injections may actually represent mesencephalocortical cells, whose axons were severed or damaged at the level of the injection sites. This argument also applies to earlier retrograde tracing studies on the same pathways (Fallon and Moore, '78; Nauta et al., '78; Fallon, '81) and represents a limit of all currently available retrograde tracing techniques. However, it must also be remembered that the distribution of mesencephalocortical neurons, as seen in the present study, corresponds only in small part to the topography of mesencephaloaccumbens and mesencephalostriatal cells (e.g., compare Figs. 3, 5, 6). This is in keeping with previous anterograde and retrograde tracing findings (Fallon and Moore, '78; Nauta et al., '78; Simon et al., '79) and supports the view that the vast majority of neurons retrogradely labeled from Acc and CPu injections do actually project to these target territories. Recently, a biochemical report by Scatton et al. ('80) showed that the hippocampal formation receives dopaminergic afferents from the VTA; this pathway is believed to cross the septal region before reaching the hippocampus (Scatton et al., '80). It is possible, therefore, that injections placed into the LS may have damaged axons of passage and caused consequent labeling of mesencephalohippocampal neurons. In this respect it must also be considered, however, that the hippocampus certainly represents a minor site of efference of the VTA neurons (Simon et al., '79) and that a precise topographical mapping of the mesencephalohippocampal pathway has not yet been performed. Very recently, Huisman et al. ('82) have shown that, at least for the FB and NY injections, the central zone of the injection site is the only area from which retrograde transport occurs. In our material, the terminal fields of the VTA ascending projections were generally largely involved by the central zone of injection areas. As an exception, the injections placed into the FC or into the CPu, two rather large structures, did not involve completely the VTA projection targets. This may have caused some reduction of the cell bodies retrogradely labeled from these two territories. In this respect, it must be considered, however, that the distribution and the number of fluorescent cells, which were seen after injections placed into the FC (ten cases), were highly reproducible, and that the CPu injections (eleven cases) were placed into different parts of this nucleus. Since the mesencephalostriatal axons are known to possess a rich terminal arborization (see Moore and Bloom, '78) it is believed that the fluorescent cells which were seen after CPu injections do represent most of the mesencephalostriatal population.

Finally, the analysis of the present data brings forth some new important problems concerning the collateralization of the VTA ascending projections. The first question arising is whether the doubly labeled cells, which have been detected in each of the seven groups of combined injections, do belong to separate and distinct subpopulations of VTA neurons or, alternatively, represent a unique subgroup of VTA cells sending long collateral branches to different terminal sites. In the first case, the total number of VTA neurons possessing long axon collaterals would be rather large as compared to the whole VTA neural population, and, in addition, the existence of specific collateralization patterns of the VTA cells may be expected. In the latter case, a relatively small subpopulation of VTA cells would send long axon collaterals to more than two target territories, while the remainder of VTA neurons may not possess long axon collaterals. The present data do not allow to answer to this question, which will require future anatomical and physiological studies. Furthermore, as pointed out earlier, most VTA neurons are catecholamine containing, but the presence of noncatecholamine-containing somata has also been demonstrated in the same region. Therefore, the question arises as to whether the VTA neurons which give off divergent axon collaterals do contain dopamine. This problem has not been investigated directly and it is open for future research; however, some indications can be obtained from data in the literature. A careful comparison of the present chartings with those obtained by means of anatomohistochemical techniques (Albanese, '82; Albanese and Bentivoglio, '82) reveals that the distribution of nondopaminergic neurons projecting to the FC, LS, and Acc does not overlap with the topography of the VTA cell population which is doubly labeled from the same areas. This consideration supports the view that the

doubly labeled cells are dopaminergic. However, some recent pharmacological studies (Agnati et al., '80; Chiodo et al., '80; Browder et al., '81) have pointed out the existence of more than one type of mesencephalic dopamine neuron. It is therefore apparent that the correlation of anatomical and pharmacological data is a matter of increasing complexity. However, it is probably from the cooperation of different methodological approaches that it will be possible to formulate a comprehensive functional model of the VTA ascending efferent systems.

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NOTE ADDED IN PROOF

A recent report by Swanson (Brain Res. Bull. *9:*321–353, 1982) came to our attention while this work was in press. The author reports data on the VTA efferent projections, which are in full agreement with the present results.