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DOPAMINERGIC AND NON-DOPAMINERGIC NEURONS IN THE VENTRAL TEGMENTAL AREA OF THE RAT PROJECT, RESPECTIVELY, TO THE CEREBELLAR CORTEX AND DEEP CEREBELLAR NUCLEI

Y. IKAI, M. TAKADA, Y. SHINONAGA and N. MIZUNO*

Department of Morphological Brain Science, Faculty of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606, Japan

Abstract—It has been suggested recently that dopamine in the cerebellum not only acts as a precursor for noradrenaline in afferent fibers supplied by locus coeruleus neurons, but also subserves an independent transmitter role in a separate neural system. The present study was initiated to investigate the possible sources for dopaminergic innervation of the cerebellum. Employing anterograde and retrograde axonal tracing with cholera toxin and a combination of fluorescent retrograde axonal tracing with Fluoro-Gold and tyrosine hydroxylase immunofluorescence histochemistry, we found in the rat that the ventral tegmental area, containing the A10 dopaminergic cell group, sends projection fibers to the cerebellum bilaterally with a slight contralateral predominance. The projections from the ventral tegmental area to the cerebellum were segregated into the dopaminergic one to the cerebellar cortex and the non-dopaminergic one to the deep cerebellar nuclei. Dopaminergic fibers projecting from the ventral tegmental area to the cerebellar cortex terminated mainly in the granular layer, additionally in the Purkinje cell layer, but not at all in the molecular layer. They were distributed predominantly in the crus 1 ansiform lobule and paraflocculus, and to a lesser extent in the crus II ansiform lobule. On the other hand, non-dopaminergic fibers projecting from the ventral tegmental area to the deep cerebellar nuclei were seen to terminate mainly in the lateral nucleus, to a lesser extent in the interpositus nucleus, but not at all in the medial nucleus. The ventral tegmental area was also observed to receive projection fibers from the lateral and interpositus cerebellar nuclei bilaterally with a contralateral predominance.

The projections from the ventral tegmental area to the cerebellum revealed in the present study might exert limbic influences upon the cerebro-cerebellar loops subserving the execution and co-ordination of voluntary movements.

Until recently, it was considered that catecholaminergic innervation of the cerebellum arises from noradrenergic neurons in the locus coeruleus.[4,17,30,47] However, many lines of pharmacological evidence[5,7,11,12,25,26] have pointed towards an indication that dopamine in the cerebellum not only acts as a precursor for noradrenaline, but also subserves an independent transmitter role in a separate neural system. This notion has further been substantiated by the immunohistochemical demonstration of dopaminergic fibers in the cerebellum using antibodies against dopamine itself.[32]

An early biochemical study[21] has described that electrolytic lesions of the ventral midbrain tegmentum, including the renowned dopaminergic cell groups[10] in the retrorubral field (RF) (A8), substantia nigra pars compacta (SNc) (A9) and ventral tegmental area (VTA) (A10), cause a marked reduction in dopamine levels in the cerebellum. A previous autoradiographic work[39] has also reported, albeit only briefly, that anterogradely labeled axon terminals occur in the cerebellum after injection of [^{3}H]leucine into the ventral midbrain regions centered on the VTA. Although these findings strongly suggest that the A8–A10 cell groups are the sites of origin of dopaminergic afferent fibers to the cerebellum, the details of such neuronal connections still remain to be established. Thus, the present study was initiated to re-investigate the possible sources for dopaminergic innervation of the cerebellum in the rat.

*To whom correspondence should be addressed.

Abbreviations: CTb, cholera toxin B subunit; FG, Fluoro-Gold; IN, interpositus cerebellar nucleus; LN, lateral cerebellar nucleus; MN, medial cerebellar nucleus; RF, retrorubral field; SCP, superior cerebellar peduncle; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

EXPERIMENTAL PROCEDURES

Eighteen male albino rats (Wistar) weighing between 280 and 320 g were used for this study. The animals were anesthetized with ether inhalation, followed by sodium pentobarbital administration (40–50 mg/kg b.wt, i.p.).

Fluorescent retrograde axonal tracing combined with tyrosine hydroxylase immunofluorescence histochemistry

Of 18 rats, 12 rats received single or multiple injections of a 4% aqueous solution of Fluoro-Gold (FG) into the cerebellum. The tracer was stereotaxically deposited unilaterally into a variety of cerebellar regions through a 1-μl Hamilton microsyringe. First, a total volume of 1.0–1.2 μl of FG was injected as widely involving both the cerebellar cortex and deep cerebellar nuclei over five to six needle penetrations (0.2 μl each; in two rats). Second, a volume

of 0.1 μl of FG was injected into the cerebellar cortex, especially into the crus I ansiform lobule or paraflocculus, in single needle penetrations (in five rats). Third, a volume of 0.05 μl of FG was injected into the deep cerebellar nuclei, especially into the lateral (LN) or interpositus (IN) cerebellar nucleus, in single needle penetrations (in five rats). Each injection was made slowly over 10 min and the injection needle was kept in place for an additional 10 min. After a survival of three to four days, the rats were deeply re-anesthetized and perfused transcardially with 300 ml of 10% formalin in 0.1 M phosphate buffer (pH 7.4). The brains were immediately removed, immersed in the same buffer containing 30% sucrose at 4°C until they sank, and then cut serially into frontal sections of 40 μm thickness on a freezing microtome.

Subsequently, the sections through the midbrain were processed for immunofluorescence histochemistry for tyrosine hydroxylase (TH) that converts tyrosine to DOPA. Briefly, they were incubated with rabbit antisera against TH[28,46] (1:1000 dilution) overnight at 4°C, followed by biotinylated goat anti-rabbit IgG (Vector, 1:200 dilution) for 2 h at room temperature and Texas Red streptavidin (Vector, 1:200 dilution) under the same condition. These immunostained sections, along with the cerebellar sections through the injection sites of FG, were mounted on to clean glass slides and then observed with a Zeiss epifluorescence microscope. An UV filter providing excitation light of approximately 360 nm wavelength was used to examine gold-emitting FG-positive cells, while a green filter providing excitation light of approximately 550 nm wavelength was used to examine red-emitting TH-immunoreactive cells.

Cholera toxin anterograde and retrograde axonal tracing

In the remaining six rats, single stereotaxic injections of cholera toxin B subunit (CTb) (List) were made unilaterally into the VTA. A 1% solution of CTb dissolved in 0.1 M phosphate buffer (pH 6.0) was deposited iontophoretically through a glass micropipette (tip diameter 25–35 μm). The driving current (positive, 2 μA, 7-s on–off cycle) was delivered for 30–60 min. The rats were allowed to survive for six to seven days, deeply re-anesthetized, and killed by transcardial perfusion with 300 ml of 10% formalin in 0.1 M phosphate buffer (pH 7.4). The brains were immediately removed, saturated with 30% sucrose in the same buffer at 4°C, and then sectioned serially in the frontal plane at 40 μm thickness on the freezing microtome. In order to visualize injected and transported CTb,[24] the sections through the injection sites and cerebellum were immunostained according to the avidin–biotin–peroxidase complex method. Briefly, they were incubated with goat antisera against CTb (List, 1:5000 dilution) overnight at 4°C, followed by biotinylated donkey anti-goat IgG (Jackson, 1:1000 dilution) for 2 h at room temperature and avidin–biotin–peroxidase complex (Vector, 1:200 dilution) under the same condition. Finally, the sections were reacted in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.04% diaminobenzidine tetrahydrochloride (Dojin), 0.003% H_2O_2 and 0.04% $NiCl_2$ for 10–20 min at room temperature.[16] They were mounted on to gelatin-coated glass slides and lightly counterstained with 0.5% Neutral Red.

RESULTS

In the first set of experiments, large multiple injections of FG into the cerebellum were made to determine the sites of origin of afferent fibers from the ventral midbrain tegmentum to the cerebellum. The injections widely involved a variety of cerebellar cortical areas and deep cerebellar nuclei (Fig. 1a). Following such cerebellar injections, a number of retrogradely labeled neuronal cell bodies were found in the ventral midbrain tegmentum bilaterally with a slight predominance of the contralateral side. The vast majority (more than 95%) of these FG-positive cells were distributed within the VTA throughout its entire rostrocaudal extent (Fig. 1b–f), while only a few FG-positive cells were encountered in the medial portions of the SNc at its rostral levels (Fig. 1b, d). No cells in the RF were retrogradely labeled with FG. After processing of TH immunohistochemistry, many neurons in the VTA and SNc displayed the intense red fluorescence induced by Texas Red. Since dopamine-β-hydroxylase that converts dopamine to noradrenaline is not detectable in any cell bodies in the ventral midbrain tegmentum,[42] TH immunoreactivity can be considered to be a determinant for the dopaminergic nature of cells in this region. All the FG-positive SNc cells were TH immunoreactive (Fig. 1b, d), and approximately 30% of the total FG-positive VTA cells were double labeled with TH antisera (Fig. 1b–f). These double-labeled cells were somewhat more frequently observed in the rostral parts than in the caudal parts of the VTA (Fig. 1b–f).

In the second set of experiments, CTb injections into the VTA were performed to examine the distribution pattern of cerebellar afferent fibers arising from the VTA. In three of six rats, the injections were successfully done to be confined to the VTA. As seen in a representative case (Figs 2a, 5a), injected CTb covered the major portion of the VTA without noticeable diffusion into surrounding structures, such as the SNc, RF, red nucleus, interpeduncular nucleus, and interfascicular nucleus. A substantial number of CTb-labeled axons emerged from the injection site to enter the superior cerebellar peduncle (SCP). Some axons were seen to cross the midline at the decussation of the SCP, leaving others uncrossed. The labeled axons were traced bilaterally via the SCP into the cerebellum. After running across the white matter, they extended to both the cerebellar cortex and deep cerebellar nuclei.

In the cerebellar cortex, anterogradely labeled axons and terminal boutons were found bilaterally with a slight predominance of the contralateral side. A moderate number of them were observed in the granular layer, a small number of them in the Purkinje cell layer, but none in the molecular layer (Fig. 3). The labeled terminals in the granular layer sometimes formed the "rosettes" reminiscent of mossy fiber endings, along with many fine varicose boutons (Fig. 3). Such anterograde labeling in the cerebellar cortex occurred sporadically in the lateral portions of the cerebellar hemisphere: predominantly in the crus I ansiform lobule and paraflocculus, and to a lesser extent in the crus II ansiform lobule (Fig. 4). No labeled axons and terminal boutons were detected in the cortical areas of the vermis and intermediate portions of the cerebellar hemisphere.

In the deep cerebellar nuclei, anterogradely labeled axons and terminal boutons were seen bilaterally with a slight contralateral predominance. They were

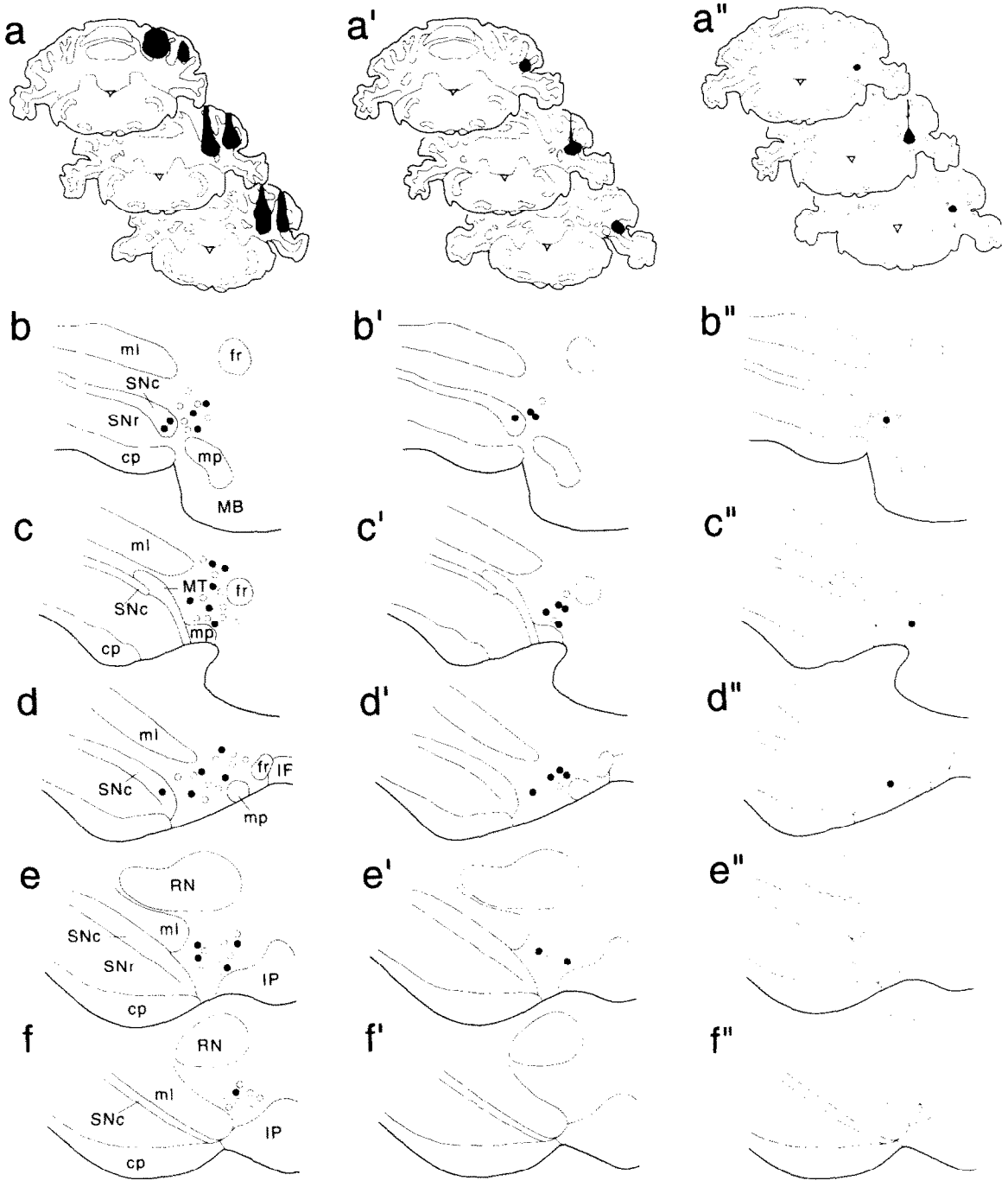

Fig. 1. Distribution of TH-immunoreactive VTA cells retrogradely labeled with FG injected into a variety of cerebellar regions. Sites of FG injections (blackened areas) in multiple cerebellar cortical areas and deep cerebellar nuclei (a), in the crus I ansiform lobule (a′), and in the LN (a″), and resulting FG-positive cells in the VTA contralateral to each injection site (b–f, b′–f′ and b″–f″, respectively). In a, a′ and a″, three frontal sections through the cerebellum are arranged rostrocaudally. In b–f, b′–f′ and b″–f″, five equidistant frontal sections through the VTA are arranged rostrocaudally. Closed circles or open circles represent cells double labeled with both FG and TH antisera or cells single-labeled with FG only, respectively. Each circle in b–f corresponds to three cells, while each circle in b′–f′ and b″–f″ corresponds to two cells. cp, cerebral peduncle; fr, fasciculus retroflexus; IF, interfascicular nucleus; IP, interpeduncular nucleus; MB, mammillary body; ml, medial lemniscus; mp, mammillary peduncle; MT, medial terminal nucleus of the accessory optic system; RN, red nucleus; SNr, substantia nigra pars reticulata.

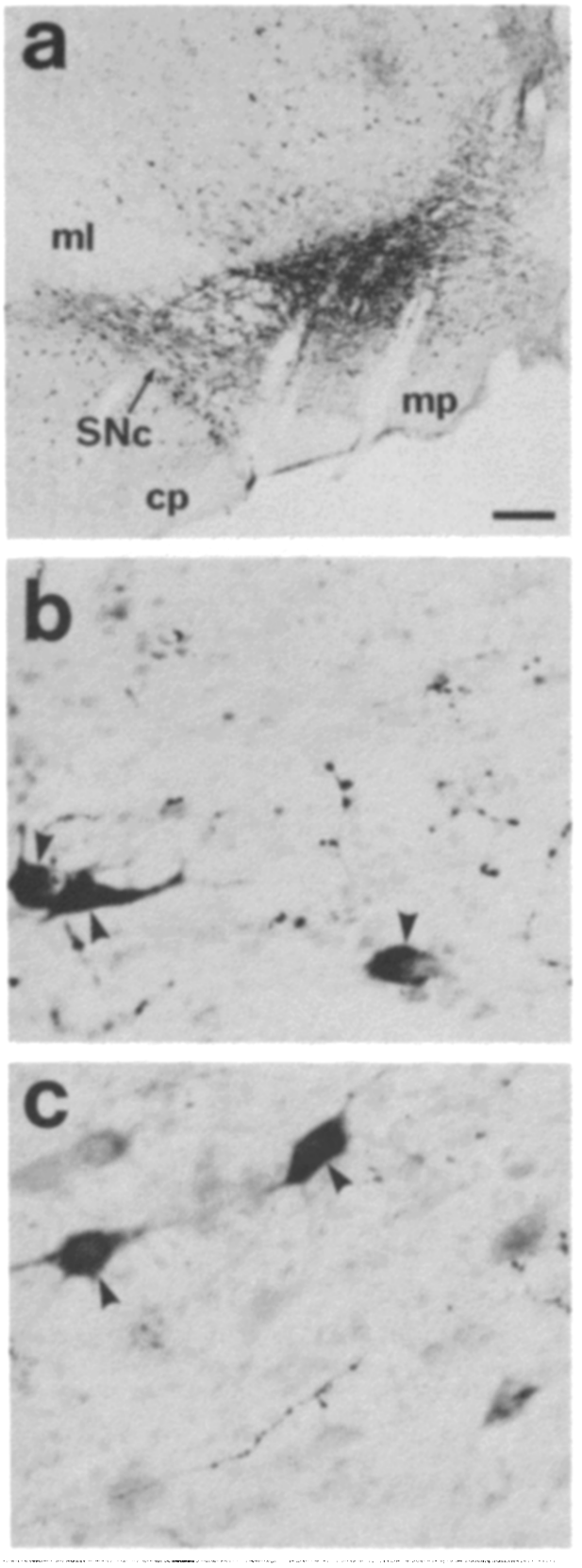

Fig. 2. A site of CTb injection in the VTA (a), and resulting anterogradely labeled axons and terminal boutons in the LN (b) and IN (c) contralateral to the injection site. Note that retrogradely labeled neuronal cell bodies are also seen in the LN and IN (arrowheads in b and c, respectively). Abbreviations are as in Fig. 1. Scale bar = 200 μm (a) and 20 μm (b, c).

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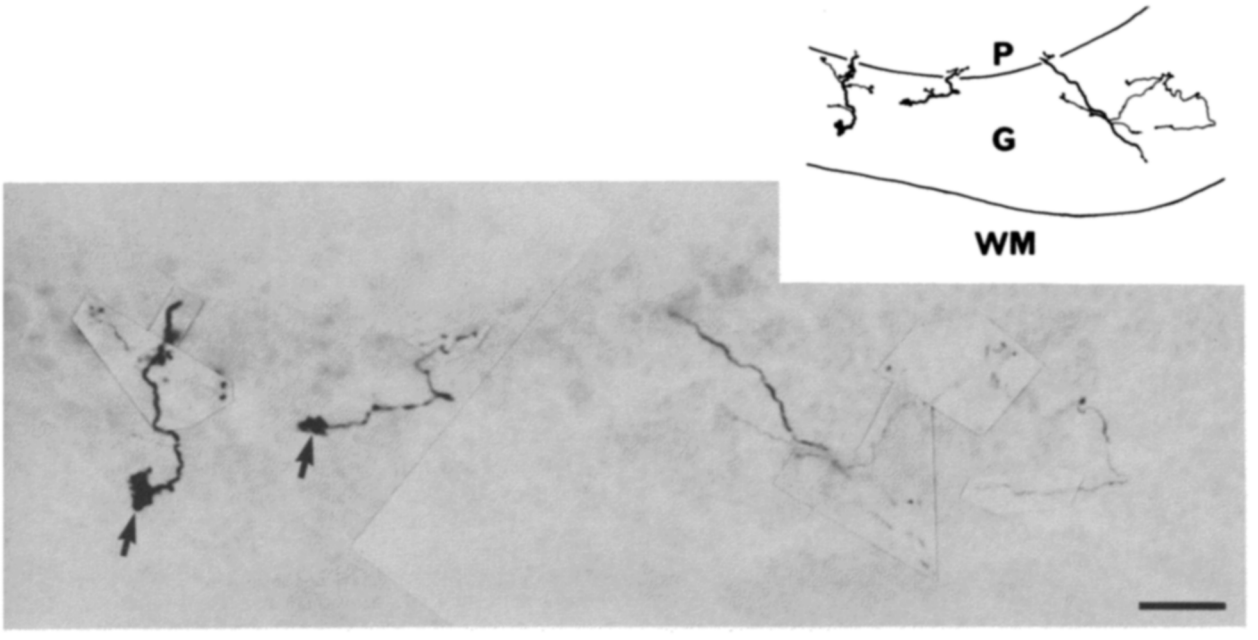

Fig. 3. Photomicrograph and camera lucida drawing (inset) of anterogradely labeled axons and terminal boutons in the crus I ansiform lobule after CTb injection into the contralateral VTA as depicted in Fig. 2a. Arrows point to "rosettes" characteristic of mossy fiber endings in the granular layer (G). P, Purkinje cell layer; WM, white matter. Scale bar = 40 μm.

located mainly in the LN (Figs 2b, 5b–e), and to a lesser extent in the IN (Figs 2c, 5b–d). No terminal labeling was found in the medial cerebellar nucleus (MN; Fig. 5c–e). The CTb-labeled axons and terminal boutons were denser in the rostral parts than in the caudal parts of the LN and IN (Fig. 5b–f).

Retrogradely labeled neuronal cell bodies were also seen in the rostral parts of the LN (Figs 2b, 5b–d) and IN (Figs 2c, 5b, d) bilaterally with a contralateral predominance. No labeled neuronal cell bodies were found in the MN (Fig. 5c–e).

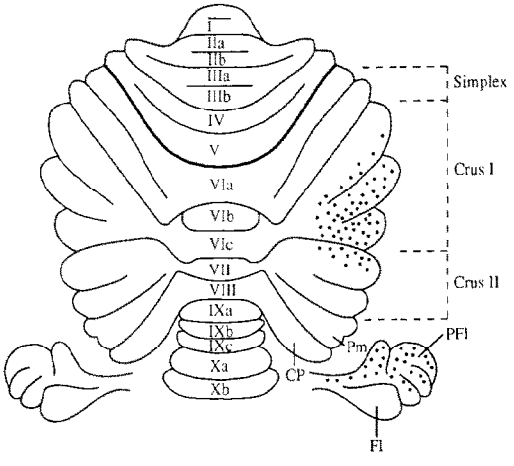

Fig. 4. Schematic diagram of the cerebellar cortex with unfolded surfaces,[22] showing the distribution of fibers and terminals (dots) arising from the VTA. CP, copula pyramis; Crus I, crus I ansiform lobule; Crus II, crus II ansiform lobule; Fl, flocculus; PFl, paraflocculus; Pm, paramedian lobule; Simplex, simple lobule.

In the third set of experiments, the same approach as in the first experimental series was employed to examine whether VTA cells projecting to the cerebellar cortex or deep cerebellar nuclei are dopaminergic or not. In three of five rats, injected FG was centered on the cortex of the crus I ansiform lobule (Fig. 1a′) or paraflocculus without spread into the deep cerebellar nuclei. As seen in a representative case (Figs 1b′–e′, 6a, a′), the injection into the cortex of the crus I ansiform lobule resulted in the occurrence of VTA cells double labeled with both FG and TH antisera. These double-labeled cells amounted to as many as 70% of the total population of FG-positive VTA cells (Fig. 1b′–f′). The double labeling was observed bilaterally with a slight predominance of the contralateral side, and was somewhat more marked in the rostral parts than in the caudal parts of the VTA (Fig. 1b′–f′). A few cells in the SNc were also double labeled with both markers (Fig. 1b′).

In two of five rats injected with FG into the deep cerebellar nuclei, injected FG was localized in the LN. As seen in a representative case (Fig. 1a″), the injection site involved almost the entire extent of the nucleus without much diffusion into the cerebellar cortex. Following such an injection, FG-positive cells were found in the VTA throughout its entire rostrocaudal extent (Fig. 1b″–f″). They were detected bilaterally with a slight contralateral predominance,

and outnumbered those seen after FG injection into the cerebellar cortex. However, the vast majority (more than 90%) of the VTA cells that were labeled with FG injected into the LN were non-TH immunoreactive (Figs 1b″–f″, 6b, b′). Similar results were obtained in a rat which was injected with FG into the confines of the IN, although FG-positive VTA cells in this rat were less numerous than those seen in the rats injected with FG into the LN.

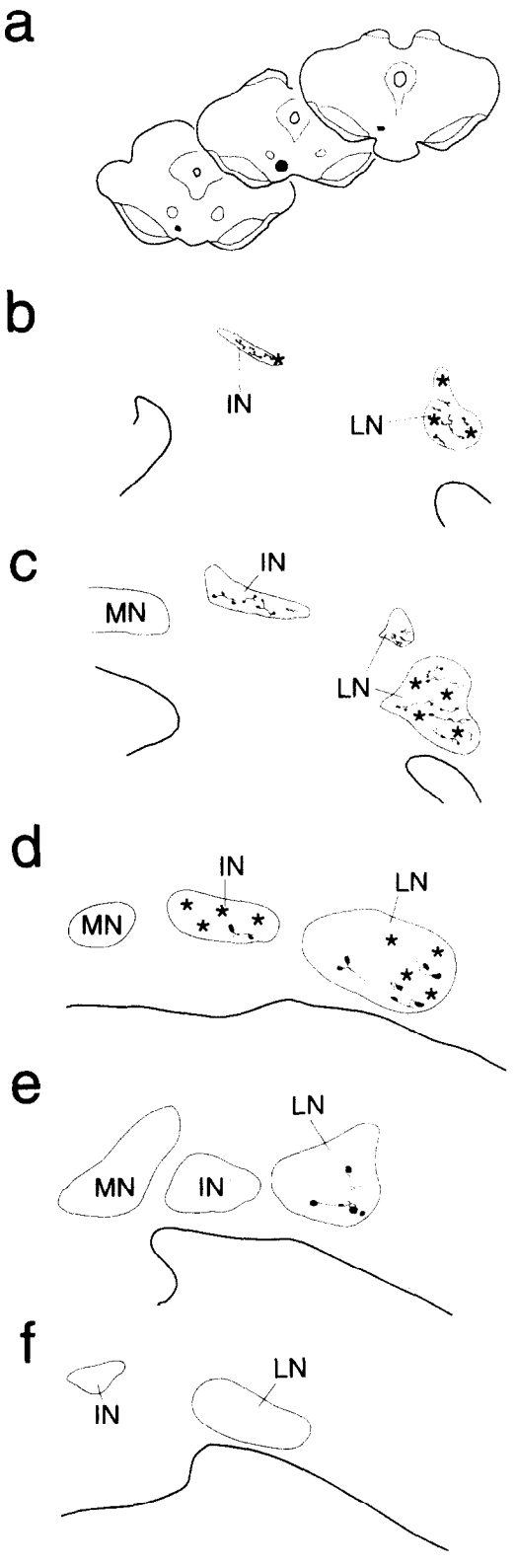

Fig. 5

DISCUSSION

The present study provides morphological evidence for direct projections from the VTA to the cerebellum in the rat. The projection fibers arise from the entire extent of the VTA, run through the SCP, and then extend to both the cerebellar cortex and deep cerebellar nuclei. The fibers separate bilaterally at the decussation of the SCP and distribute to the cerebellum bilaterally with a slight contralateral predominance. The VTA neurons sending their axons to the cerebellum appear to be classified into two groups. The fluorescent retrograde axonal tracing combined with TH immunofluorescence histochemistry reveals that at least 70% of the total VTA cells projecting to the cerebellar cortex are dopaminergic, whereas more than 90% of the total VTA cells projecting to the deep cerebellar nuclei are non-dopaminergic. If the observed slight involvement of the cerebellar cortex in FG injection into the deep cerebellar nuclei could, to some degree, account for the retrogradely labeled VTA cells that were TH immunoreactive (dopaminergic), then the ratio of the non-dopaminergic population to the total cells projecting to the deep cerebellar nuclei would become even higher. Indeed, no available work has so far indicated the existence of dopaminergic afferent fibers to the deep cerebellar nuclei. The VTA projection seems to be more massive towards the deep cerebellar nuclei than towards the cerebellar cortex. However, this is not yet conclusive, because the sporadic distribution of VTA-derived axon terminals in the cerebellar cortex makes it difficult to count all the cells projecting to the cerebellar cortex with the fluorescent retrograde axonal tracing technique employed in the present study.

The dopaminergic fibers projecting to the cerebellar cortex terminate mainly in the granular layer, additionally in the Purkinje cell layer, but not at all in the molecular layer. The dopaminergic axon terminals in the granular layer occasionally constitute the "rosettes" characteristic of mossy fiber endings.

Fig. 5. A site of CTb injection (blackened areas) in the VTA (a), and the distribution of resulting anterogradely labeled axons and terminal boutons (lines and dots) and retrogradely labeled neuronal cell bodies (asterisks) in the deep cerebellar nuclei contralateral to the injection site (b–f). In a, three equidistant frontal sections through the VTA are arranged rostrocaudally. In b–f, five equidistant frontal sections through the deep cerebellar nuclei are arranged rostrocaudally. Note that the anterograde and retrograde labeling occurs in both the LN and IN, but not in the MN.

However, recent immunohistochemical work[32] using dopamine antibodies has indicated that dopaminergic fibers in the cerebellar cortex may be distributed in all cortical layers, in particular abundance in the molecular layer. The discrepancy could be explained by postulating that dopamine immunoreactivity might represent not only dopamine as an independent transmitter, but also dopamine as a precursor for noradrenaline. In fact, early catecholamine histofluorescence experiments,[4,15,18,47] sometimes in combination with loading of catecholaminergic fibers with α-methyl-noradrenaline or 6-hydroxytryptamine, have suggested that noradrenergic fibers are located in all cerebellar cortical layers with the highest density in the molecular layer. Two types of autoradiographic studies[4,35,38] have also reported that cerebellar noradrenergic fibers arising almost exclusively from the locus coeruleus terminate predominantly within the molecular layer, and to a lesser extent within the Purkinje cell layer: intracisternal injection of [^{3}H]noradrenaline results in radiolabeling of axon terminals along the apical dendrites of Purkinje cells,[4] and after injection of [^{3}H]proline into the locus coeruleus, anterogradely labeled axon terminals in the cerebellar cortex are restricted to Purkinje cell soma and proximal dendrites.[35,38]

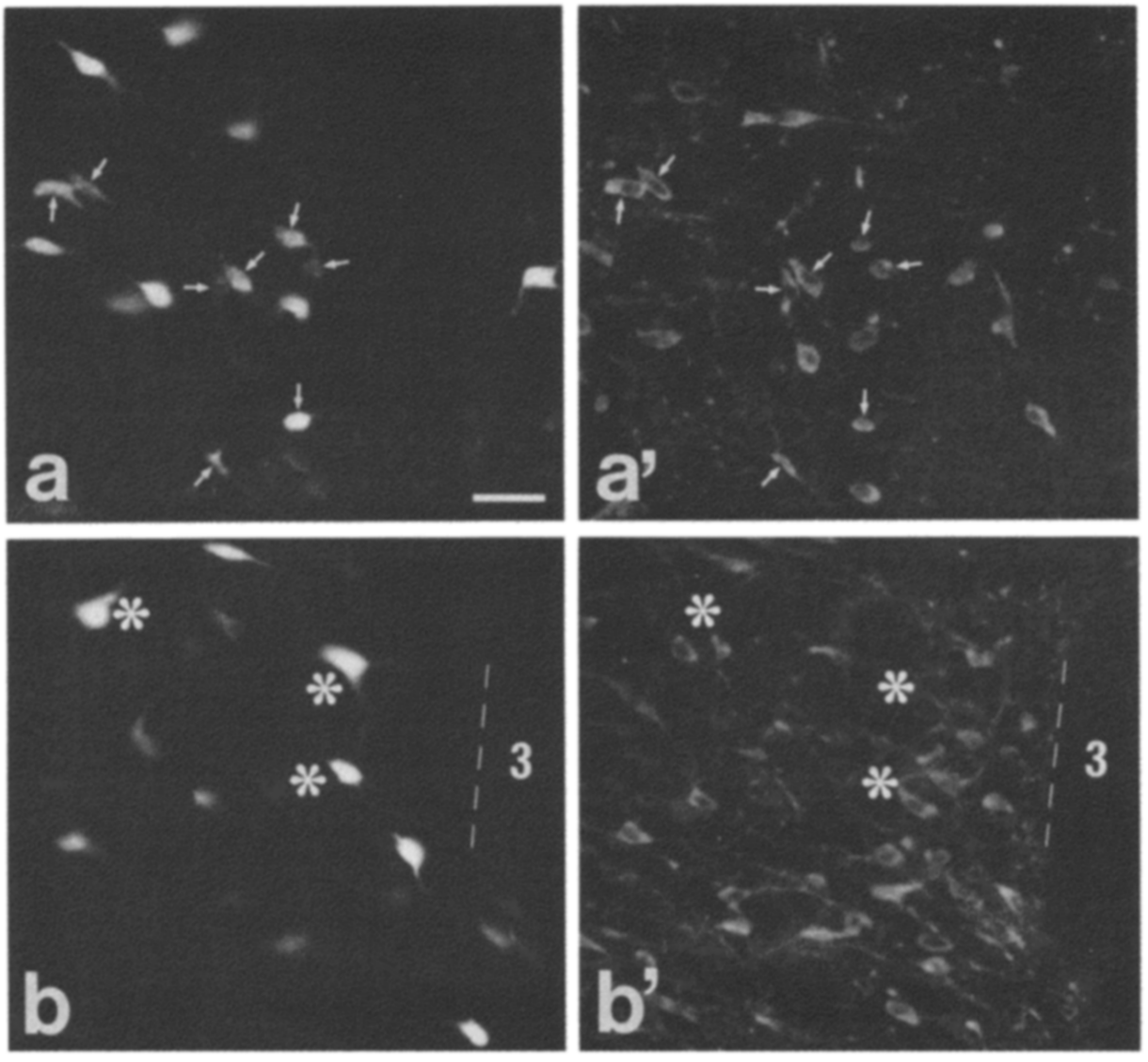

Fig. 6. TH immunoreactivity in VTA cells retrogradely labeled with FG injected into the crus I ansiform lobule as depicted in Fig. 1a′, and into the LN as depicted in Fig. 1a″. (a) FG-positive cells after the injection into the contralateral crus I ansiform lobule. (a′) TH-immunoreactive cells in the same field as a. (b) FG-positive cells after the injection into the contralateral LN. (b′) TH-immunoreactive cells in the same field as b. Arrows in a and a′ point to cells double labeled with both FG and TH antisera. Asterisks in b and b′ indicate identical blood vessels. 3, oculomotor nerve root. Note that many double-labeled cells are seen after the injection into the crus I ansiform lobule, whereas no cells are double labeled after the injection into the LN. Scale bar = 40 μm.

Thus, it is likely that two distinct catecholaminergic fiber systems exist in the cerebellum: the dopaminergic fibers originating from the VTA and the noradrenergic fibers originating from the locus coeruleus. While the noradrenergic fibers in the cerebellar cortex are seen in both the vermis and hemisphere of the cerebellum,[35,47] the VTA-derived dopaminergic fibers in the cerebellar cortex identified here are distributed in the lateral portions of the cerebellar hemisphere: mainly in the crus I ansiform lobule and paraflocculus, and to a lesser extent in the crus II ansiform lobule. Since cholecystokinin is often co-localized in dopaminergic cells in the VTA,[19] the VTA projection to the cerebellar cortex might contain this neuropeptide as well as dopamine, although the cerebellum has been reported to contain only a small amount of cholecystokinin.[3,49]

Recently data concerning the differential localization of dopamine receptors in the cerebellum have been accumulated. Dopamine receptor binding, especially for the D_1 subtype, occurs in both the molecular and granular layers,[12,25] while dopamine receptor mRNAs for both the D_1 and D_2 subtypes are present in the granular layer only,[6,25] thus suggesting that cerebellar dopamine receptors are synthesized in the granular layer, perhaps in granule cell bodies, and are either transported through granule cell axons to the molecular layer to form presynaptic receptors, or remain in granule cell bodies to constitute postsynaptic receptors. Given that these dopamine receptors appear to be widely distributed over the cerebellar cortex,[6,12,25] the possible existence of sources other than the VTA (A10) for dopaminergic innervation of the cerebellum cannot as yet be excluded. As evidenced by the present results, however, the major dopaminergic cell groups in the RF (A8) and SNc (A9) seem to send only a few or no projection fibers to the cerebellum. It should also be noted here that the dopaminergic projection from the VTA to the cerebellar cortex identified in the present study terminates in both the granular and Purkinje cell layers, where the mRNA of the newly characterized D_3 type of dopamine receptor has been reported to be expressed.[6] The D_3 dopamine receptors that have been shown to follow closely the A10 projections[6] possibly function, together with the D_1 dopamine receptors, as postsynaptic receptors on granule and Purkinje cells, because DARPP-32, a phosphoprotein enriched in dopaminoceptive neurons (particularly D_1 dopamine receptor-containing ones), has been found in both cerebellar cells.[31]

The present study further reveals that VTA cells send projection fibers to the deep cerebellar nuclei. These projection fibers terminate predominantly in the LN, to a lesser extent in the IN, but not at all in the MN. Such a distribution pattern corresponds to that of VTA-derived axon terminals in the cerebellar cortex that are preferentially distributed in the lateral portions of the cerebellar hemisphere. In contrast to the dopaminergic nature of VTA cells projecting to the cerebellar cortex, the VTA cells projecting to the deep cerebellar nuclei are primarily non-dopaminergic. On the basis of the fact that glutamate decarboxylase that converts glutamate to GABA is enriched in both cell bodies in the VTA and axon terminals in the deep cerebellar nuclei,[27] GABA is considered to be a likely transmitter for VTA neurons sending their axons to the deep cerebellar nuclei. The dopaminergic vs non-dopaminergic nature of the VTA-cerebellar projections suggests that the afferent fibers to the cerebellar cortex and deep cerebellar nuclei probably take their origins from two distinct neuronal populations in the VTA. However, it is generally accepted that at least part of the afferent fibers to each deep cerebellar nucleus comprise axon collaterals of those to the overlying cerebellar cortical area.[20,36,48] From this point of view, a non-dopaminergic population (albeit only less than 30%) of the VTA cells projecting to the cerebellar hemisphere might issue axon collaterals to the LN or IN, although the close proximity of the axonal courses towards these target structures makes it impossible to verify the possibility using fluorescent retrograde double labeling. It should be mentioned here, however, that despite a wide variety of output targets for the VTA, including the anterior limbic cortex, nucleus accumbens, lateral septum, amygdala and lateral habenula,[2,9,14,29,39,41] the paucity of divergent collateral projections from individual cells to more than one terminal field appears to be a general feature of the output organization of VTA cells.[1,13,23,29,41,43-45]

It is also indicated in the present study that projection fibers from the deep cerebellar nuclei terminate in the VTA, thus constituting a reciprocal connection between these structures. In accordance with the distribution pattern of VTA-derived afferent fibers in the deep cerebellar nuclei, the cells of origin of the projections are located in the LN and IN bilaterally with a contralateral predominance, but not in the MN. Such direct projections from the deep cerebellar nuclei to the VTA have previously been reported by two horseradish peroxidase[33,39] and a silver impregnation[40] studies. According to Perciavalle *et al.*,[33] the projection fibers from the LN and IN terminate not only in the VTA, but also widely in other ventral midbrain tegmental regions, including the RF, SNc and red nucleus.

CONCLUSION

The functional roles of the VTA-cerebellar projections still remain obscure. The two separate populations of VTA cells, dopaminergic ones projecting to the cerebellar cortex and non-dopaminergic ones projecting to the deep cerebellar nuclei, would rather be considered to act as independent functional units than as a functionally synchronized unit. The VTA receives input from limbic system-associated

structures, including the anterior limbic cortex, nucleus accumbens, amygdala and lateral habenula.[29,34,39] In view of the fact that the projection fibers from the VTA to the cerebellum terminate in the so-called "pontocerebellum", the VTA–cerebellar projections might exert limbic influences upon the cortico-ponto-cerebellar loops which have been known to play important roles in the execution and co-ordination of voluntary movements.[8,20,37]

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