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MIDBRAIN DOPAMINERGIC NEURONS IN THE MOUSE: CO-LOCALIZATION WITH CALBINDIN-D_{28K} AND CALRETININ

C.-L. LIANG, C. M. SINTON and D. C. GERMAN*

Department of Psychiatry, University of Texas, Southwestern Medical Center, Dallas, TX 75235-9070, U.S.A.

Abstract—The calcium-binding proteins Calbindin-D_{28k} and calretinin are co-localized with dopamine in some of the midbrain dopaminergic neurons in the rat and monkey; the present study sought to examine the pattern of co-localization in the mouse. Double immunofluorescence staining procedures were used for tyrosine hydroxylase (a dopaminergic cell marker) and Calbindin-D_{28k} or calretinin. Midbrain dopaminergic neurons were examined at four rostrocaudal levels, and the percentage of cells that contained both tyrosine hydroxylase and either of the two calcium-binding proteins was determined in nucleus A8 (retrorubral field), nucleus A9 (substantia nigra pars compacta, pars reticulata and pars lateralis) and nucleus A10 (nucleus paranigralis, ventral tegmental area, interfascicular nucleus, central linear nucleus). The two calcium-binding proteins were distributed similarly in midbrain dopaminergic neurons in the several nuclear groups that comprise nuclei A8, A9 and A10. The calcium-binding proteins were found in the majority (50–100%) of nucleus A10 neurons, whereas in nuclei A8 and A9 (except for the substantia nigra pars lateralis) less than 40% of the cells contained either calcium-binding protein. The pattern of co-localization in the mouse is similar to that reported for the rat and monkey.

The calcium-binding proteins mark the population of midbrain dopaminergic neurons that are less vulnerable to degeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: calcium-binding proteins, immunocytochemistry, tyrosine hydroxylase.

Calbindin-D_{28k} and calretinin are calcium-binding proteins that are widely distributed in the CNS of mammals, and in the rat brain they are localized primarily in non-overlapping neuronal populations.[1,2,6,13,20,24,30,37,38,39,40,41,43,44,48] Calbindin-D_{28k} and calretinin are EF-hand homolog proteins, in the same family as calmodulin, parvalbumin and the S100s, which bind Ca^{2+} with dissociation constants in the micromolar range, and they are modulated by stimulus-induced increases in cytosolic free Ca^{2+}.[19,36]

The dopaminergic (DA) neurons in the midbrain have been found to contain Calbindin-D_{28k} and/or calretinin. In the human, monkey and rat, Calbindin-D_{28k} is localized in many midbrain DA neurons.[7,13,15] In the rat, some midbrain DA neurons contain both Calbindin-D_{28k} and calretinin.[43]

In Parkinson's disease, and in animals treated with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; which produces alterations in intracellular Ca^{2+} as one component of its toxicity), there is a preferential loss of those midbrain DA neurons that lack Calbindin-D_{28k}[15,23,25,54] or calretinin.[31] These observations suggest that Calbindin-D_{28k} and/or calretinin may play a neuroprotectant role in these DA neurons. Recent immunohistochemical investigations have demonstrated that Calbindin-D_{28k} gene expression may be a critical factor which determines neuronal survival in the substantia nigra during Parkinson's disease.[15,25] It is possible that reduced Calbindin-D_{28k} gene expression in key brain areas leads to excitotoxic vulnerability and Ca^{2+}-mediated neuronal degeneration.[23]

Although there have been no studies of the distribution of the two calcium-binding proteins in the mouse brain, the mouse is an important species in which to examine this issue because it represents an animal model of Parkinson's disease.[18,26,51] The C57BL/6 mouse strain is very susceptible to MPTP-induced degeneration of midbrain DA neurons.[5,17,18,23] In the present study, we used double-labeling immunofluorescence methods to visualize simultaneously tyrosine hydroxylase (TH; the rate-limiting enzyme in the synthesis of dopamine in the midbrain neurons) and Calbindin-D_{28k} or calretinin in the midbrain of the C57BL/6 mouse

*To whom correspondence should be addressed.

Abbreviations: CLi, central linear nucleus; DA, dopaminergic; IF, interfascicular nucleus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBP, nucleus parabrachialis; PBS, phosphate-buffered saline; PBST, PBS containing 0.3% Triton X-100; PN, nucleus paranigralis; RRF, retrorubral field; SNC, substantia nigra pars compacta; SNL, substantia nigra pars lateralis; SNR, substantia nigra pars reticulata; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

strain. We also examined the FVB/N mouse strain, because this strain is ideally suited for transgenic studies.[53]

EXPERIMENTAL PROCEDURES

Animals

Three adult male C57BL/6 mice (Taconic Farms, Germantown, NY, U.S.A.) and three adult male FVB mice (Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) were used for immunocytochemical staining (20–25 g). The mice were deeply anesthetized with Nembutal (120 mg/kg, i.p.), and perfused through the ascending aorta with 0.1 M phosphate-buffered saline (PBS; pH 7.4, 2–3 min) and 10% neutral buffered formalin (10 min). The brains were blocked in a coronal plane and postfixed in formalin for 5–10 h. After immersion in a 20% sucrose solution for 12 h or longer, 20-μm-thick sections were cut on a freezing microtome.

Immunocytochemistry

In order to choose representative areas for double immunostaining, tissue sections were first stained with a polyclonal antibody against TH (Eugene Tech International, Allendale, NJ, U.S.A.), using the peroxidase–antiperoxidase method. Details of the staining procedure have been published previously.[10] The immunostained sections were used to map the locations of TH-containing cells on a computer-imaging system. Details of the computer-imaging procedure have been published previously.[10,14] The sections adjacent to those that were stained for TH were processed for simultaneous two-color fluorescence immunocytochemistry. The sections were rinsed with 0.1 M PBS containing 0.3% Triton X-100 (PBST) for 30 min, and then incubated overnight with a mixture of two primary antibodies that were raised from different species. The antibodies include rabbit Calbindin-D_{28k} antiserum (1 : 1000, from Dr A. M. Iacopino), mouse monoclonal Calbindin-D_{28k} antibody (1 : 150, SWant, Bellinzona, Switzerland), sheep anti-TH antibody (1 : 150, Pel-Freez) and rabbit calretinin antiserum (1 : 1000, SWant). The TH antibody provided very distinct immunostaining of the midbrain DA neurons without background staining. The Calbindin-D_{28k} antibody has been shown not to cross-react with calretinin, and has been used previously in our laboratory, among others.[13,23] The calretinin antibody has also been shown not to cross-react with Calbindin-D_{28k}.[47]

After incubation in the primary antibodies, sections were washed three times in PBS (10 min per wash), and then incubated with a mixture of fluorescein-conjugated donkey anti-mouse IgG (1 : 50, Jackson, West Grove) or fluorescein-conjugated donkey anti-sheep IgG (1 : 50, Jackson) and Texas Red-conjugated donkey anti-rabbit IgG (1 : 50, Jackson) for 3 h. The sections were washed twice in PBST and twice in PBS, and then mounted on gelatin-coated slides and air-dried. The sections were coverslipped using a phenylenediamine/glycerin/PBS mounting medium. Immunocytochemically stained sections were viewed with a Leitz fluorescence microscope equipped with appropriate filter systems, which included: N2.1, a 515–560-nm bandpass filter used for exciting fluorescein, and a 580-nm longpass barrier filter to limit emission to red; L3, a 450–490-nm bandpass filter used for exciting Texas Red, and a 515–560-nm bandpass barrier filter to limit emission to green; and G/R, a filter system for green and red, a bandpass 490/20-nm and 675/30-nm filter for exciting both fluorescein and Texas Red, and a bandpass 525/20-nm and 635/30-nm barrier filter to limit emission to both green and red.

For all six brains, the extent of co-localization of TH with Calbindin-D_{28k} or calretinin was determined. All brains were examined by two people, and a semi-quantitative method was used to determine the percentage of cells that contained both TH and a calcium-binding protein. In one brain from a C57BL/6 mouse, computer-imaging procedures[10,14] were used to quantify the number of cells that contained TH alone, TH and Calbindin-D_{28k}, and TH and calretinin from rostral to caudal in four representative midbrain regions.

RESULTS

Sections for analysis

Sections from four rostrocaudal levels were chosen for immunocytochemical staining (Fig. 1). Level 1 is at the middle portion of the mammillary nucleus, which contains the DA neurons in the substantia nigra pars compacta (SNC), substantia nigra pars reticulata (SNR) and rostral ventral tegmental area (VTA) (Fig. 1A). Level 2 is at the caudal portion of the mammillary nucleus, which contains the same regions as in level 1, and also the interfascicular nucleus (IF; Fig. 1B) just medial to the fasciculus retroflexus. Level 3 is at the rostral interpeduncular nucleus (Fig. 1C), which contains the substantia nigra pars lateralis (SNL) and nucleus paranigralis (PN). Level 4 is at the caudal interpeduncular nucleus, and contains the caudal extent of nucleus A10, the central linear nucleus (CLi) and the retrorubral field (RRF) (Fig. 1D).

Calbindin-D_{28k} and calretinin immunoreactivity are present throughout the midbrain DA neuron areas. The cell distributions were very similar in both C57BL/6 and FVB/N mouse strains. The following data will therefore be described only in the C57BL/6 strain, as an example of the results applying to both strains.

There are substantial differences in the intensity of Calbindin-D_{28k} and calretinin immunoreactivity in different DA cells. Using the filter system that allowed simultaneous visualization of red and green fluorescence (G/R filter), the TH-containing cells were green (using fluorescein isothiocyanate), the calcium-binding protein-containing cells were red (using Texas Red) and the double-labeled cells were shades of green–yellow (Fig. 2). If the cells contain relatively high concentrations of TH and calcium-binding protein, they appear yellow. If the cells contain relatively high concentrations of TH and only faint amounts of calcium-binding protein, they appear apple green. For the latter cells, very faint red immunostaining is observed using the L3 filter system. These observations indicate that DA neurons can contain different quantities of Calbindin-D_{28k} or calretinin.

Co-localization of Calbindin-D_{28k} and tyrosine hydroxylase

The magnitude of co-localization of TH with the two calcium-binding proteins is based upon semi-quantitative analysis in six mice, and quantitative

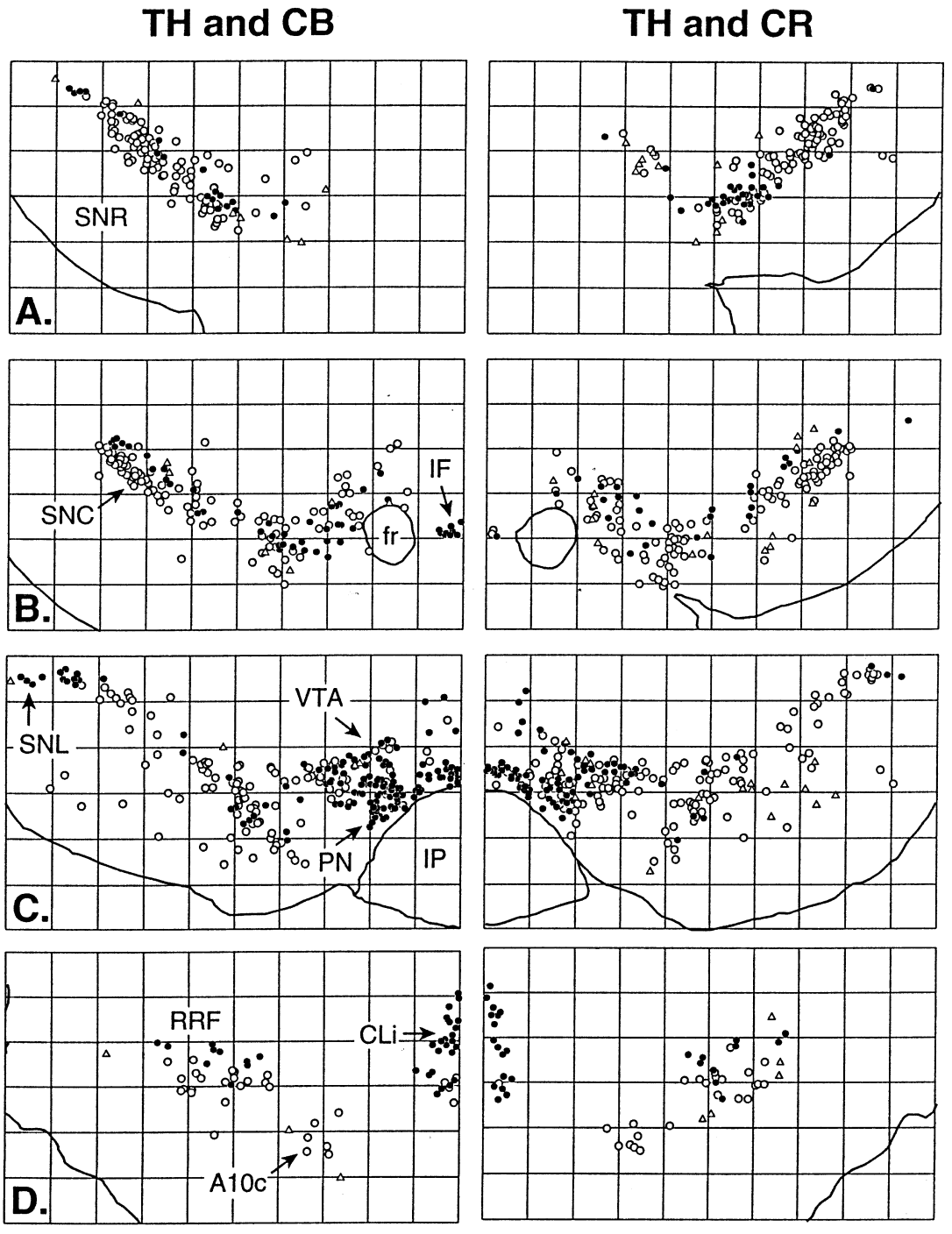

Fig. 1. Computer-generated maps of midbrain cell locations in representative sections from rostral (A) to caudal (D) in the C57BL/6 mouse. Open circles represent TH-immunoreactive cells, closed circles represent TH-immunoreactive cells that also contain a calcium-binding protein, and open triangles represent cells that only contain a calcium-binding protein. The tissue sections are 20 μm thick. Grid lines are separated by 0.2 mm, and the abbreviations are defined in the text.

measurements in one of these mice (Fig. 1 and Table 1). The two methods gave converging data.

In nucleus A8, in the RRF, about 30% of the DA neurons contain Calbindin-D_{28k}. As can be observed in Fig. 1, the double-labeled cells are confined to the dorsal portion of the RRF, whereas the ventral portion of the RRF contains DA neurons that mainly lack Calbindin-D_{28k}.

Depending upon the specific portion of nucleus A9, from 0% to 100% of the DA neurons contain Calbindin-D_{28k}. In the SNC, from rostral to caudal (Fig. 1A–C), about 20% of the DA cells contain

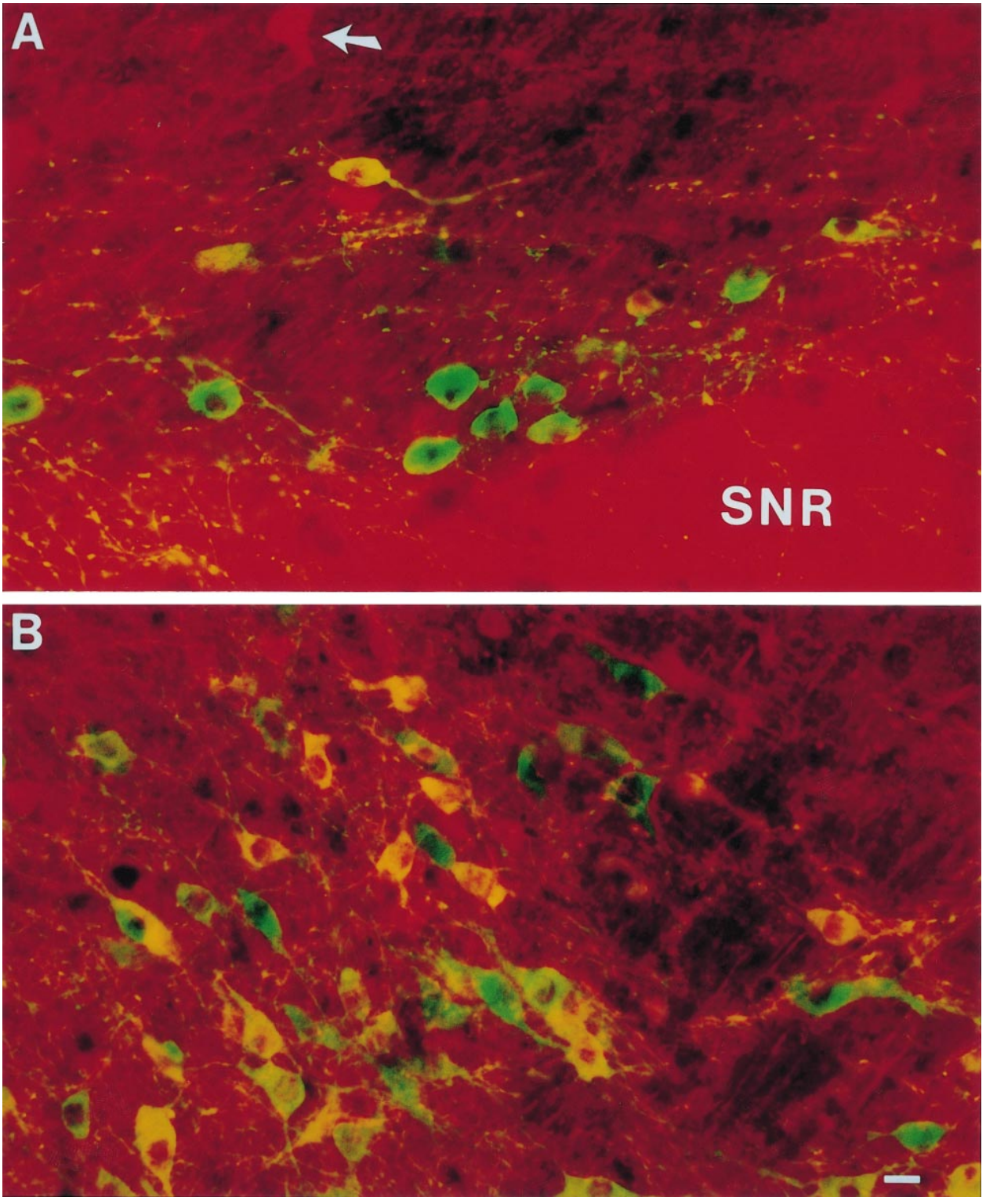

Fig. 2. Double immunofluorescent labeling of TH and Calbindin-D_{28k}. (A) In the SNC several TH-immunoreactive neurons (green) are located above the SNR. In the dorsal portion of the SNC, cells often contain both TH and Calbindin-D_{28k} (yellow). The arrow illustrates a cell that only contains Calbindin-D_{28k} (red). (B) In the VTA there are numerous cells that contain both TH and Calbindin-D_{28k} (yellow), but some of the larger multipolar neurons contain only TH. Scale bar = 12 μm.

Table 1. Percentage of dopaminergic neurons that contain Calbindin-D_{28k} or calretinin in a C57BL/6 mouse

	A8	A9			A10				
	RRF	SNC	SNR	SNL	VTA	CLi	PN	IF	A10c
No. of TH neurons* (unilateral)	28	248	15	5	145	23	30	24	7
% Double stained (Calbindin-D_{28k} + DA)	32.3	21.7	0.0	100.0	55.0	85.7	97.0	100.0	0.0
% Double stained (calretinin + DA)	40.0	14.7	0.0	100.0	42.8	94.4	66.7	82.6	0.0

These data represent a numerical analysis of data presented in Fig. 1.
*The number of TH-immunostained neurons, unilaterally, in each of the four tissue sections in which the structures exist.

Calbindin-D_{28k}. The cells that contain Calbindin-D_{28k} are mainly medium- or small-sized (18×12 μm) multipolar and fusiform neurons (Fig. 3B), and are located in the medial and dorsal portions of the SNC. The TH-immunoreactive neurons that lack Calbindin-D_{28k} have large multipolar somata (25×20 μm–20×15 μm), are round to oval in shape, and have a nucleus that is unstained for TH (Fig. 3A). In the SNL, more than 90% of the TH-positive cells contain Calbindin-D_{28k} immunoreactivity. Both the TH and Calbindin-D_{28k} immunoreactivities are very weak. Throughout the rostrocaudal extent of the SNR, less than 5% of the TH-containing cells are Calbindin-D_{28k} positive.

In nucleus A10, which includes the VTA (defined here to include cells in the nucleus parabrachialis pigmentosus (PBP)), CLi, PN, IF and A10c, from less than 10% to 100% of the DA cells contain Calbindin-D_{28k}. Numerous double-immunostained neurons can be seen in the VTA region (Fig. 3C, D). In the VTA, about 50% of the DA cells contain Calbindin-D_{28k}. In the IF, PN (situated immediately lateral to the interpeduncular nucleus) and CLi, nearly all of the DA cells (80–100%) contain Calbindin-D_{28k} immunoreactivity. These DA cells have smaller somata than DA cells in the SNC and PBP. There are many cells that are only TH immunoreactive on the dorsal edge of the VTA. All of them have large somata and clear unstained nuclei, like the SNC cells that only contain TH. About 50% of the TH + Calbindin-D_{28k} neurons in nucleus A10 exhibit intense Calbindin-D_{28k} immunofluorescence, and they are located in all subnuclei.

Co-localization of calretinin and tyrosine hydroxylase

The distribution of TH + calretinin neurons in the midbrain is similar to that of TH + Calbindin-D_{28k} neurons (Figs 1, 4). It is important to note that there are many calretinin-immunoreactive cells around the midbrain DA complex that do not contain TH. There are many calretinin-immunoreactive cells dorsal to the VTA, in the medial portion of the SNC, and along the midline above the IF.

In nucleus A8, the distribution of TH + calretinin neurons is similar to that of the TH + Calbindin-D_{28k} neurons. Overall in the RRF, about 40% of the DA neurons contain calretinin. In the dorsal portion of the RRF, however, many of the DA neurons contain calretinin but few double-labeled neurons are located in the ventral portion of the nucleus (Figs 1D, 4E, F).

In nucleus A9, most TH + calretinin neurons are located in the medial SNC, and the cells are small- to medium-sized multipolar neurons (Fig. 1A). Some TH + calretinin neurons were located in the dorsal–lateral portion of the SNC; these were typically small, fusiform-shaped neurons (Fig. 4A, B). Nearly all SNL DA neurons contain calretinin immunostaining (Fig. 1C), but both the TH and calretinin stainings are of weak intensity. In the SNR there are no DA neurons that contain calretinin (Fig. 1A–C).

In nucleus A10 regions, many of the subnuclei contain high proportions of calretinin-containing neurons. In the VTA, about 40% of the DA neurons contain calretinin (Figs 1C, 4C, D). In CLi, PN and IF, 65–95% of the DA cells contain calretinin. However, in nucleus A10c none of the DA cells contains calretinin. Comparing the intensity of calretinin immunoreactivity, DA neurons with strong calretinin immunostaining only appear on the dorsal portion of the VTA.

Co-localization of Calbindin-D_{28k} and calretinin

In nucleus A10 regions (including the IF, PN and CLi), and in the SNL, most neurons are Calbindin-D_{28k} + calretinin double-immunostained. We observed different intensities of Calbindin-D_{28k} and calretinin immunoreactivity in different neurons. The medial SNC appears to form an extension of the VTA because most of these cells have double-staining characteristics similar to those in the VTA. In the SNC, a few fusiform-shaped calretinin-immunoreactive neurons do not contain Calbindin-D_{28k}, and some small Calbindin-D_{28k}-immunoreactive neurons do not contain calretinin. In the dorsal half of the RRF, about 50–60% of the immunoreactive neurons are Calbindin-D_{28k} + calretinin double-labeled, about 20% of the immunoreactive neurons are only Calbindin-D_{28k} positive, and 25% are only calretinin positive.

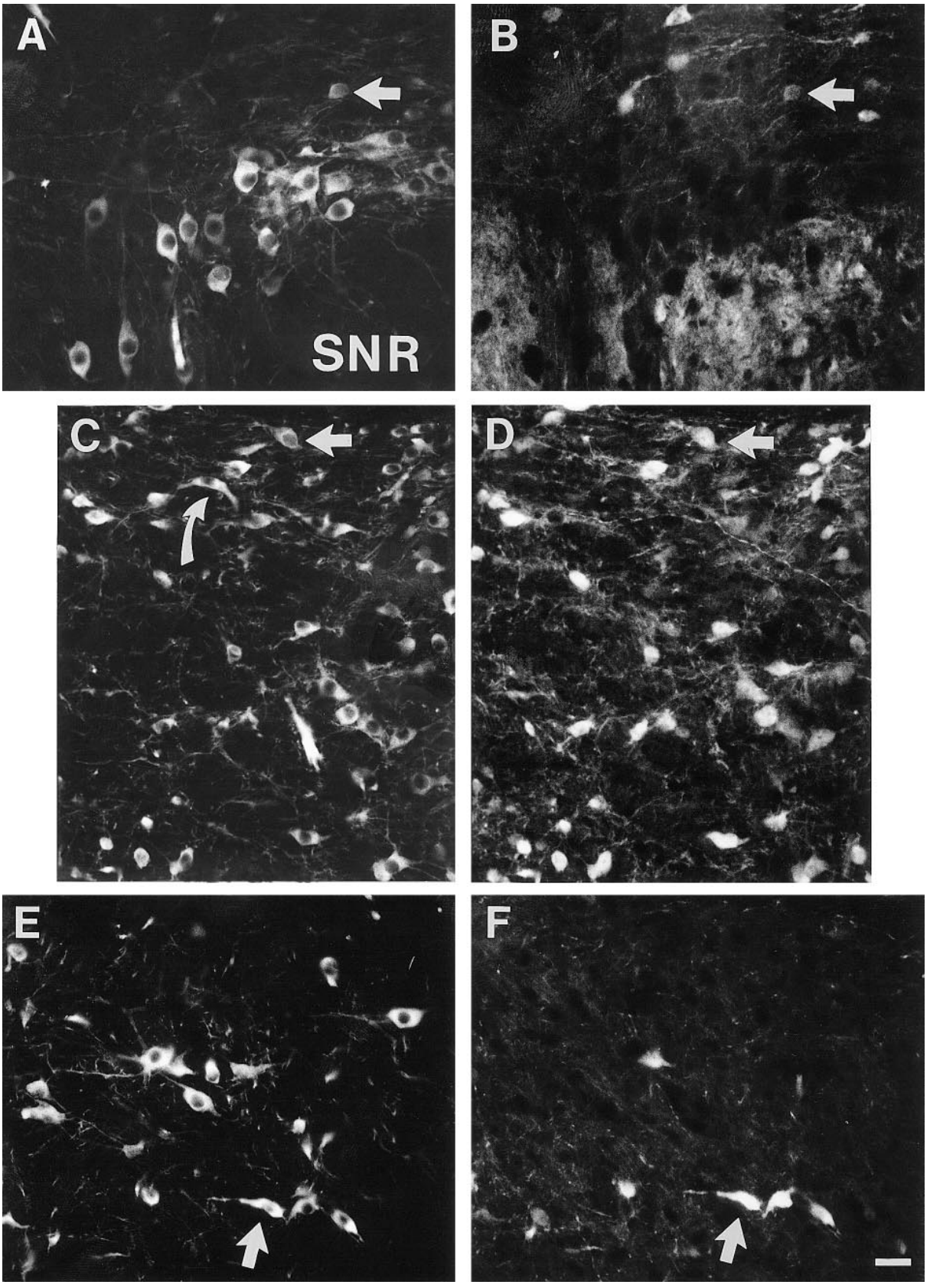

Fig. 3. Double-immunostained sections for TH and Calbindin-D_{28k}. (A, C, E) Sections stained for TH; (B, D, F) the same sections stained for Calbindin-D_{28k}. (A, B) SNC cells, above the SNR: most of the cells only stain for TH, but some of the dorsal SNC cells stain for both TH and Calbindin-D_{28k}. (C, D) Staining in the VTA: many VTA cells stain for both TH and Calbindin-D_{28k}. (E, F) Staining in the RRF: only a few cells stain for both TH and Calbindin-D_{28k}. Arrows point to cells stained in corresponding right and left panels, and provide a reference point to compare the two photomicrographs. Scale bar = 22 μm.

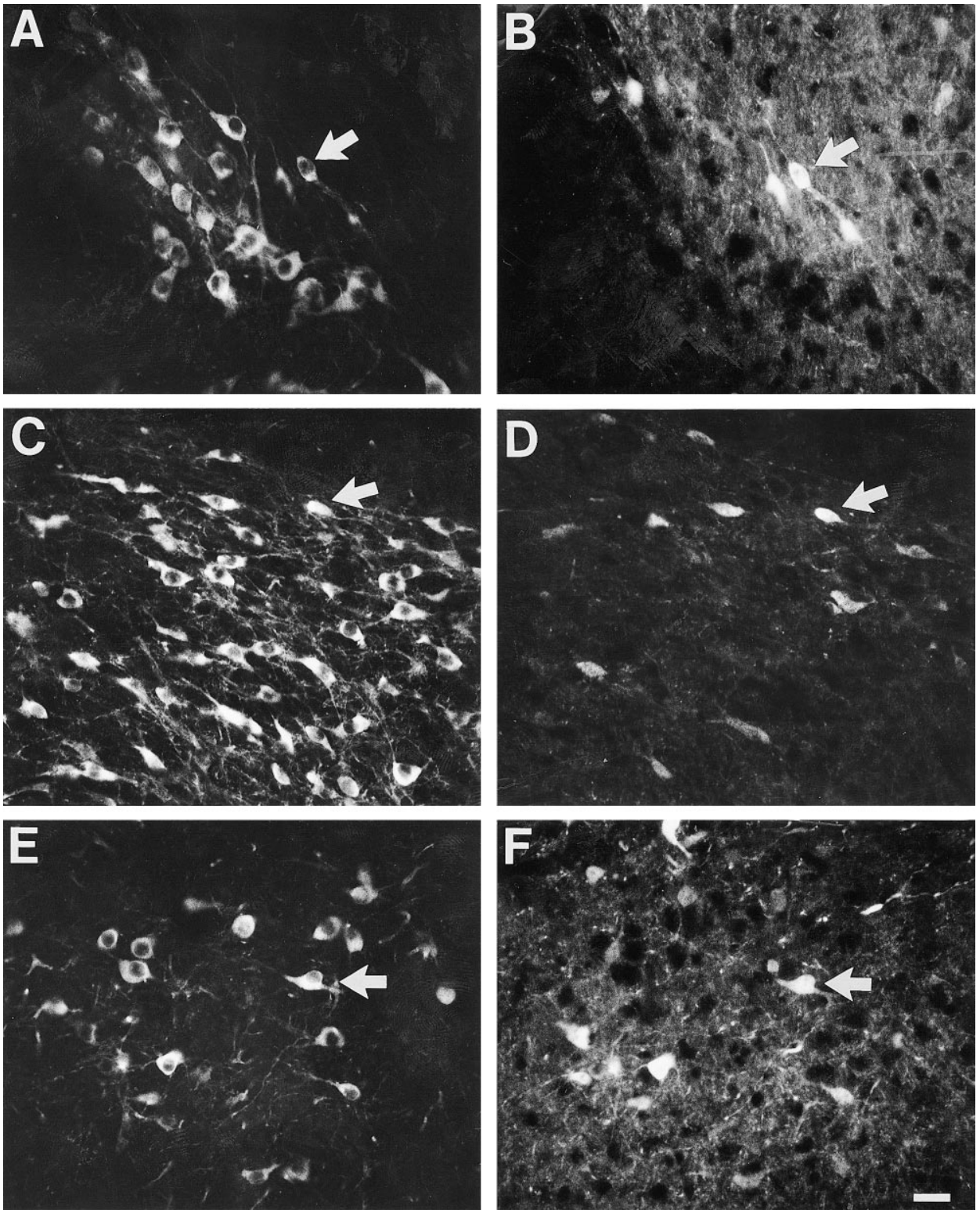

Fig. 4. Double-immunostained sections for TH and calretinin. (A, C, E) Sections stained for TH; (B, D, F) the same sections stained for calretinin. (A, B) SNC cells: most of the cells only stain for TH, but some of the dorsal SNC cells stain both for TH and calretinin. (C, D) Staining in the VTA. (E, F) Staining in the RRF. Note that a small portion of the nucleus A8 DA neurons contains calretinin. Arrows point to cells stained in corresponding right and left panels, and provide reference points to compare the two photomicrographs. Scale bar = 22 μm.

DISCUSSION

Characteristics of dopaminergic neurons that contain Calbindin-D_{28k} or calretinin

Most of the TH + Calbindin-D_{28k} and TH + calretinin double-labeled neurons are medium- to small-sized, multipolar and fusiform-shaped neurons. In contrast, the midbrain DA neurons that lack the calcium-binding proteins Calbindin-D_{28k} and calretinin, especially in the SNC and VTA, are large-sized multipolar neurons, often greater than 20 μm in the long axis. These neurons are located mainly in the SNC and the dorsal portion of the VTA (within the PBP), and some at the lateral extent of the CLi. Gerfen *et al.*[8,9] used anterograde axonal tracing with *Phaseolus vulgaris*-leucoagglutinin, combined with immunocytochemical staining for TH and Calbindin-D_{28k}, and autoradiographic localization of μ-opiate receptors to study the mesostriatal DA projections in the rat. They found that the DA neurons that contain Calbindin-D_{28k} in the VTA, dorsal tier of the SNC and RRF correspond to the DA neurons that project to the striatal matrix compartment; these fibers have thin diameters and fewer axonal varicosities. The DA neurons that do not express Calbindin-D_{28k} (those in the ventral tier of the SNC and SNR) are distributed in a pattern that matches the origin of the DA projection to the striatal patches; these fibers have slightly thicker axonal diameters, with more frequent varicosities. The large DA neurons which do not contain Calbindin-D_{28k} are likely to be the neurons innervating striatal patches, and the medium-sized DA neurons which contain Calbindin-D_{28k} and/or calretinin are neurons which innervate the matrix of the ventromedial striatum, including the nucleus accumbens.

The present study provides data in the mouse concerning the proportions of midbrain DA neurons that contain Calbindin-D_{28k} and/or calretinin. Up to 95% of the DA neurons in midline regions of nucleus A10 (such as PN, CLi, IF) contain Calbindin-D_{28k} and calretinin. In A10 subnuclei further from the midline, 40–80% of the DA neurons contain Calbindin-D_{28k} and calretinin; these include the VTA and PBP. These data from the mouse are similar to data from the rat.[24,42] However, the greatest discrepancy involves the co-localization of calretinin with TH in the SNC and SNR; mouse nigral DA neurons infrequently contain Calbindin-D_{28k} or calretinin (not counting the medial SNC cells), but approximately 70% of rat SNC and SNR DA neurons contain calretinin.[42] The calretinin data of Isaacs and Jacobowitz,[24] in the rat, are more in line with the present data in the mouse. Why the two studies in the rat are different is unclear.

The distribution of cells labeled for both TH and calcium-binding proteins is similar to that of the midbrain DA neurons that contain the neuroactive peptides cholecystokinin and neurotensin.[21,22,49,50] The midbrain DA neurons that contain Calbindin-D_{28k} and calretinin are most frequently found in nucleus A10 regions and in the SNL portion of nucleus A9. In the rat, it has been demonstrated that Calbindin-D_{28k} is localized within the same population of midbrain DA neurons that contains cholecystokinin and neurotensin.[11] The calcium-binding proteins may play a role in the regulation of intracellular calcium levels to modulate the co-release of peptides with dopamine.

Nuclear organization of calcium-binding proteins

Since the identification of DA neurons in midbrain nuclei A8, A9 and A10 by Dahlstrom and Fuxe,[3] the establishment of boundaries between the three nuclear groups has been problematic.[4,52] The borders between nuclei A9 and A10 have most often been established using the rat brain atlas of Paxinos and Watson;[35] this was done for both the rat[12] and mouse[32] midbrain DA neurons. In the present study, many of the DA neurons in the medial SNC contain calcium-binding proteins in similar proportions to the DA neurons in the VTA region. Therefore, the medial SNC neurons may represent part of nucleus A10, and not part of nucleus A9. A similar conclusion has been drawn in the human brain regarding the localization of Calbindin-D_{28k} in the medial SNC.[28,29] These neurochemical data would support the inclusion of cells currently positioned in the medial SNC (nucleus A9) with cells in the lateral VTA (nucleus A10). Consistent with this proposal, the medial SNC DA cells in the rat have been shown to project to similar limbic regions as other nucleus A10 cell groups.[52]

The organization of midbrain DA neurons in the mouse is similar to that in humans. In the mouse substantia nigra there are three tiers of cells (see Fig. 1), beginning dorsally with the SNC DA neurons that contain the calcium-binding proteins, then the SNC DA neurons that lack the two calcium-binding proteins, and finally the SNR DA neurons that lack the calcium-binding proteins. This is similar to the τ, β and σ tiers of the human substantia nigra as described by Olszewski and Baxter.[34] The Calbindin-D_{28k}- and calretinin-containing DA neurons begin rostrally in the dorsal, medial and lateral portions of the SNC and VTA regions, whereas the ventral SNC and SNR DA neurons do not contain the calcium-binding proteins. This pattern continues to the caudal-most portion of the midbrain DA cell complex, where in nucleus A8 the double-labeled cells reside in the dorsal portion of the RRF, and the ventral portion of the RRF and nucleus A10c do not contain double-labeled cells. The DA neurons that contain the calcium-binding proteins may represent a separate functional system from the DA neurons that lack the calcium-binding proteins.

Calcium-binding proteins and neurotoxicity

The midbrain DA neurons that contain the calcium-binding proteins are less vulnerable to MPTP toxicity.[25,26] Three and six hours after treatment with MPTP (20 mg/kg), in the C57BL/6 mouse, there were 42% and 128% increases in Calbindin-D_{28k} protein concentrations in the VTA, respectively, as determined by western blot analysis.[33] The elevation in Calbindin-D_{28k}, in response to MPTP, may protect the VTA DA neurons from neurotoxicity. The SNC neurons, most of which lack Calbindin-D_{28k} and calretinin, are most vulnerable to degeneration in Parkinson's disease, and to MPTP toxicity.[14,15,16,25,26] However, MPTP causes degeneration of some of the DA neurons that contain Calbindin-D_{28k}, at a time when DA neurons that lack Calbindin-D_{28k} are still preserved.[23,26] Thus, Calbindin-D_{28k} is not the only factor that determines which cells survive MPTP toxicity. The activity of the dopamine transporter, which transports the MPTP toxin into the DA neurons, also appears to play a role in determining which DA neurons are vulnerable to MPTP toxicity. The mRNA for the dopamine transporter is low in midbrain DA cell regions that are spared MPTP-induced degeneration in the mouse,[45] and there is a positive correlation between the location of the vulnerable cells and the location of cells that contain high levels of dopamine transporter mRNA.[46] Cells that contain low dopamine transporter activity and calcium-binding proteins should be least vulnerable to degeneration in Parkinson's disease and following MPTP treatment.

CONCLUSIONS

The calcium-binding proteins Calbindin-D_{28k} and calretinin are localized within a subpopulation of midbrain DA neurons in the mouse. Midline nuclei, within nucleus A10, and the SNL contain the highest proportion of double-labeled neurons (labeled both for TH and one or both of the calcium-binding proteins). The midline nuclei include the IF, PN and CLi. Within nucleus A8, less than half of the DA neurons contain Calbindin-D_{28k} and/or calretinin, and within nucleus A9 (SNC and SNR), less than 20% of the cells contain either calcium-binding protein. This pattern of co-localization in the mouse is similar to that found previously in the rat. The buffering of intracellular calcium by Calbindin-D_{28k} and/or calretinin may serve several functions for the midbrain DA neurons, such as regulating the co-release of neuroactive peptides (cholecystokinin, neurotensin) with dopamine, and protecting the neurons from MPTP neurotoxicity.

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