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Immunocytochemical identification of proteins involved in dopamine release from the somatodendritic compartment of nigral dopaminergic neurons

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Abstract

We examined the somatodendritic compartment of nigral dopaminergic neurons by immunocytochemistry and confocal microscopy, with the aim of identifying proteins that participate in dopamine packaging and release. Nigral dopaminergic neurons were identified by location, cellular features and tyrosine hydroxylase immunoreactivity. Immunoreactive puncta of vesicular monoamine transporter type 2 and proton ATPase, both involved in the packaging of dopamine for release, were located primarily in dopaminergic cell bodies, but were absent in distal dopaminergic dendrites. Many presynaptic proteins associated with transmitter release at fast synapses were absent in nigral dopaminergic neurons, including synaptotagmin 1, syntaxin1, synaptic vesicle proteins 2a and 2b, synaptophysin and synaptobrevin 1 (VAMP 1). On the other hand, syntaxin 3, synaptobrevin 2 (VAMP 2) and SNAP-25-immunoreactivities were found in dopaminergic somata and dendrites Our data imply that the storage and exocytosis of dopamine from the somatodendritic compartment of nigral dopaminergic neurons is mechanistically distinct from transmitter release at axon terminals utilizing amino acid neurotransmitters.

Keywords

dopamine; substantia nigra; somatodendritic; exocytosis; dopamine release proteins

Parkinson's disease, a movement disorder involving the basal ganglia, results primarily from degeneration of the dopaminergic (DAergic) projection from substantia nigra (SN) to striatum (Birkmayer and Hornykiewicz, 1976). The underlying nigrostriatal pathway arises from large midbrain DAergic neurons situated in the SN pars compacta (SNc). Laterally extending dendrites emitted by the DAergic perikarya intermingle in the SNc and vertical dendrites extend ventrally into the adjacent pars reticulata (SNr) (Dahlström and Fuxe, 1964; Juraska et al., 1977; Wassef et al., 1981, Tepper et al., 1987).

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Previous studies have shown that dopamine (DA) is released both by the nigrostriatal axonal terminals within striatum and by DAergic somata and dendrites in the midbrain (Björkland and Lindvall, 1975; Groves et al., 1975; Geffen et al., 1976; Nieoullon et al., 1977; Rice et al., 1994, Jaffe et al., 1998; Chen and Rice, 2001). The requisite conditions for DA release at these two sites differ, however. Striatal DA release shows a steep dependence on extracellular calcium (Ca^{2+}) concentration ($[Ca^{2+}]_o$) and is undetectable when $[Ca^{2+}]_o$ falls below 1.0 mM, whereas somatodendritic release persists in 0.5 mM $[Ca^{2+}]_o$ and plateaus above 1.5 mM (Chen and Rice, 2001). However, somatodendritic release is prevented by extracellular cadmium (Cd^{2+}), a non-specific blocker of voltage-gated Ca^{2+} channels and attenuated by a Ca^{2+} chelator (BAPTA-AM) (Jaffe et al., 1998; Patel et al., 2009), indicating that Ca^{2+} entry and an increase in intracellular Ca^{2+} are required. Furthermore we have recently shown that somatodendritic DA release is facilitated by mobilization of Ca^{2+} from intracellular stores (Patel et al., 2009). Another important distinction is that DAergic terminals in striatum show typical presynaptic aggregates of agranular vesicles (Pickel et al., 1996; Nirenberg et al., 1997), whereas DAergic somata in SNc lack these aggregates (Nirenberg et al., 1996), as do DAergic dendrites within SNr (Wassef et al., 1981, Groves and Linder, 1983 but cf. Wilson et al., 1977). Nonetheless, there is evidence for quantal DA release from DAergic somata in the SNc (Jaffe et al., 1998). These findings suggest that, although mechanistically distinct, both terminal and perikaryal DA release involve Ca^{2+}-dependent exocytosis.

It is well known that neurotransmitter release by vesicle exocytosis depends on a multistage process (reviewed in Südhof, 2004), each step of which is dependent on an interlocking network of presynaptic proteins distributed among different sites, including the synaptic vesicle and at the active zones of the plasma membrane where vesicle exocytosis occurs. Parenthetically, axonal DAergic release sites may be differently organized than the vast majority of CNS synapses, in that at least some DAergic junctions in the striatum lack an apposed post-synaptic ending (Beaudet and Descarries, 1978). Moreover, DAergic axonal synapses are completely absent in the SNc (Juraska et al., 1977; Wassef et al., 1981), and only sparse DAergic dendrodendritic synapses have been reported there (Wassef et al., 1981; Groves and Linder, 1983). Dopamine thus reaches its targets by diffusion, a process called volume transmission (Agnati et al., 1986; Rice, 2000). Even though DA release sites in the somatodendritic compartment lack a synaptic structure they nevertheless resemble classical synapses in utilizing a Ca^{2+}-dependent exocytotic mechanism for DA release. We will therefore use the neutral term ‘DA release protein’ to refer to the proteins involved in DA exocytosis.

The identities of canonical presynaptic proteins have been established primarily from studies of fast amino acid transmitter release. Whether the same or different proteins are found within DAergic somata and dendrites remains largely unknown and is the main experimental question of the present study. Previous studies (Bergquist et al., 2002; Fortin et al., 2006) showed, by pharmacology and immunocytochemistry, that the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein, SNAP-25, was present in the somatodendritic compartment of nigral DAergic neurons and the presence of a synaptobrevin was inferred, suggesting the presence of exocytotic machinery in DAeregic perikarya and dendrites. In the present study we used immunocytochemistry to identify proteins involved in Ca^{2+}-dependent exocytosis of DA in SN DAergic somata and dendrites. Our goal was to test the hypothesis that differences exist in the molecular organization of DAergic somatodendritic release sites relative to those of fast synapses. We found that although many vesicular and plasma membrane proteins commonly encountered in synaptic endings of fast synapses were absent, certain isoforms of these proteins were present in DAergic somata and dendrites. Our findings thus contribute to a growing understanding of the unique characteristics of DA release from the somatodendritic compartment.

Experimental Procedures

Animals

Young adult guinea pigs (male, Hartley, 150–250g) were obtained from Charles River Laboratories (Wilmington, MA). All animal handling procedures were in accordance with National Institutes of Health guidelines and were approved by the New York University School of Medicine Animal Care and Use Committee.

Guinea pigs were deeply anesthetized with an intraperitoneal injection of Nembutal (sodium pentobarbital at 50 mg/kg), then perfused transcardially with phosphate-buffered saline (PBS: 9 g NaCl/L in 10 mM phosphate buffer, pH 7.3) followed by freshly prepared paraformaldehyde (4%) in PBS. The brain was then removed and immersed in the same fixative for 1 h. A coronal block of midbrain was dissected free and washed in PBS for 30 min before being placed for 16–24 h at 4°C in 30% sucrose in PBS. Frozen coronal sections through the midbrain were cut (at 20 μm), mounted on slides, dried for 1 h at 37 °C, then stored frozen until used for immunocytochemistry.

Immunocytochemistry

Slides were washed 3 times for 7 min each (3 × 7 min) in PBS and 30 min in blocking solution (10 ml PBS, containing 0.1 g bovine serum albumin, 30 μL Triton-X 100 and 100 μL 10% (w/v) Na azide), then incubated in a mixture of primary antibodies for 16–20 h at room temperature. Following further 3 × 7 min washes in PBS, sections were exposed to secondary antibodies, Alexa 488 (Invitrogen, Carlsbad, CA) and Cy3 (Jackson Immunoresearch, West Grove, PA) for 2 h. After a final 3 × 7 min washes in PBS, sections were coverslipped inVectaShield (Vector Labs, Burlingame, CA).

Antibodies and their specificity

A list of the antibodies used, including their sources and effective dilutions, is provided in Table I. [Table I **about here**] The numbers that sometimes follow a name (e.g., synaptotagmin 1,2) indicate the isoforms recognized by the antibody we tested. We found that omission of the primary antibodies resulted in no staining. We tested all the commercially available immunogenic peptides, and found invariably that preadsorption of the primary antibody with its immunogenic peptide abolished immunostaining; these findings are documented in the Figures. For the remaining antibodies utilized, we relied on published studies and/or the manufacturer's descriptions of specificity, based either on abolition of immunostaining following immunogen preadsorption, or Western blots showing that the antibody recognized a band of appropriate molecular size.

Data Acquisition and Analysis

Fluorescent images were obtained with a Nikon PM 800 confocal microscope equipped with a digital camera controlled by the Spot software program (Diagnostic Instruments, Sterling Heights, MI). Digital images were acquired separately from each laser channel, then recombined. Digital files were processed with deconvolution software (AutoQuant Imaging, Watervliet, NY). Adobe Photoshop 7.0 was used for further processing of digital images. Any adjustments to brightness and contrast were made uniformly to all parts of the image.

We assessed the distribution of immunoreactivity (ir) within SN DAergic perikarya and dendrites for each antibody tested by obtaining z-axis confocal scans through the tissue. For DAergic perikarya the scans were at 2.0 μm intervals and for dendrites, at 1.0 μm intervals, using a 100x objective throughout, for which the depth of field is estimated to be 0.41 μm. This estimate is derived from the formula $d_{tot} = (\lambda_o\, n/NA^2) + (n/M \times NA)e$, where d_{tot} is the total depth of field, λ_o is the wavelength of illuminating light (540 nm), n is the refractive index

of the medium (immersion oil, 1.479), NA is the numerical aperture (1.4) and e is the smallest distance (0.5 μm) that can be resolved by a detector splaced in the image plane of the microscope objective where lateral magnification is M (100) (cf. Nikon at http://www.microscopyu.com/tutorials/java/depthoffield/index.html)

Results

A. General features of SNc and SNr

The SNc is characterized by a high density of DAergic somata as well as a dense network of overlapping DAergic dendrites, as seen when the midbrain is immunostained for tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis (Fig. 1). The DAergic perikarya in SNc tend to be ovoid, with a long axis of 20–30 μm. Each cell body emits multiple dendrites that extend either laterally within the SNc or ventrally into SNr. Within SNr, DAergic perikarya are sparse (Wassef et al., 1981), but DAergic dendrites are prominent, sometimes running in bundles towards the ventral surface of the midbrain (Fig. 1).

Because SNc and SNr also contain abundant non-DAergic cells and processes (Nair-Roberts et al., 2008), our sole criterion for associating a particular protein with a DAergic perikaryon or process was the colocalization of its immunostaining with TH-ir. The use of TH-ir is advantageous in that it extends throughout the DAergic cell, filling the cell body, dendrites and axons, including the finest processes. In contrast, the immunostaining of the proteins we examined was punctate, except for syntaxin 1, whose immunostaining pattern is a mixture of puncta embedded in a continuous matrix (cf. Fig. 3). The general distribution we obtained with an anti-syntaxin antibody is in agreement with earlier studies (Ruiz-Montasell et al., 1996; Goutan et al., 1999), which documented that syntaxin 1-ir is diffusely distributed throughout the neuropil of many rat brain regions, including SNr.

B. Vesicular monoamine transporter 2 in SN DAergic somata

The vesicular monoamine transporter type 2 (VMAT2) transports DA into synaptic vesicles or other intracellular compartments participating in DA release. In the SN, somatodendritic DA release is minimal after VMAT2 inhibition (Elverfors and Nissbrandt, 1991; Rice et al., 1994; Heeringa and Abercrombie, 1995). Prior EM studies showed that SNc DAergic perikarya contain VMAT2, associated primarily with the smooth endoplasmic reticulum, but not organized into vesicle clusters (Nirenberg et al., 1996). We utilized an anti-VMAT2 antibody to gain an impression of the general distribution of VMAT2 in SNc and SNr. VMAT2-ir is distributed primarily as puncta in the perikaryal cytoplasm (Fig. 2a–c), with the highest density observed in a ring surrounding the nucleus. Additional VMAT2-ir puncta are located closer to the perikaryal surface but their density adjacent to the plasma membrane is low (Fig. 2c,d) With one exception, VMAT2-ir was noted only at the initial portions of DAergic dendrites, falling to a very low density at more distal locations (Fig. 2d), including throughout the SNr (Fig 2e). The exception is the DAergic dendrite from which the axon branches (Tepper et al., 1987). VMAT2-ir extends along this process (Fig. 2 f, g), reflecting the fact that VMAT2 is synthesized in the cell body and transported to the axon terminals in striatum where it is reported to be associated with DAergic axons and their synaptic terminals (Nirenberg et al., 1997).

The finding that VMAT2-ir is essentially absent in distal DAergic dendrites was tested further by examining the distribution of proton (vacuolar) ATPase (v-ATPase) within DAergic neurons (Fig. 2h–l). This proton pump is intrinsic to catecholaminergic vesicles and is responsible for establishing the proton gradient that energizes VMAT2-dependent transport (Eiden et al., 2004). Our results are illustrated in Fig. 2h–l. Similar to VMAT2-ir, v-ATPase-ir was seen as regions of patchy staining within DAergic perikarya (Fig. 2h–j), but was absent from DAergic dendrites. Preadsorption of the v-ATPase antibody with its immunogenic

peptide resulted in a complete loss of v-ATPase-ir (Fig. 2k–l). Together these data suggest that the packaging and storage of DA for release differs in dendrites within SNr from that observed in DAergic somata and striatal axon terminals.

C. Presynaptic proteins not associated with SN DAergic somata

Synaptic vesicles contain a large number of intrinsic proteins (reviewed in Südhof, 2004) among which either synaptic vesicle proteins 2a or 2b (sv2a, sv2b) isoforms are invariably present. However, we found that both sv2a- and sv2b-ir were absent in DAergic somata (Fig. 3a,c), although present throughout the surrounding midbrain tissue, including in clusters immediately adjacent to the DAergic perikarya. Sv2a- and sv2b-ir were abolished following preadsorption of these primary antibodies by their respective immunogenic peptides (Fig. 3b,d). These data are consistent with previous studies indicating that the SN DAergic somatodendritic compartment lacks conventional synaptic vesicles (Nirenberg et al., 1996).

DAergic perikarya also were negative for staining with antibodies against three additional synaptic vesicle proteins: synaptophysin (Fig. 3e), the Ca^{2+} sensor, synaptotagmin 1,2 (Fig. 3f), and synaptobrevin 1, also called vesicle-associated membrane protein 1 (VAMP1) (Fig. 3h). We similarly found no immunostaining for the plasma membrane protein, syntaxin 1 (Fig. 3g), a so-called SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) involved in the fusion of synaptic vesicles with the plasma membrane. It is noteworthy that all of the presynaptic proteins we examined (Fig. 3) were found in the neuropil surrounding the DAergic somata, presumably indicating their association with some or all of the diverse terminals making synapses onto the DAergic somata (Nair-Roberts et al., 2008). The majority of these inputs arise in other basal ganglia nuclei and are GABAergic or glutamatergic (Lee and Tepper, in press).

Given that agranular vesicle accumulations were reported in presumed DAergic dendrites within SNc (Wilson et al., 1977) we extended our search for proteins involved in vesicle exocytosis to DAergic dendrites, even for those proteins not found in DAergic perikarya (see above and Fig. 3). We examined dendritic profiles in both SNc and SNr and our results were uniformly negative (not illustrated), i.e., we found no evidence for the presence of any of the above mentioned presynaptic proteins in DAergic dendrites.

D. DA release proteins

DAergic neurons of the SNc are not completely devoid of release proteins, however. Indeed, three were found to be associated with DAergic somata: syntaxin 3, SNAP-25 and synaptobrevin 2 (VAMP2) (Fig. 4). Syntaxin 3 and SNAP-25 are SNARE proteins localized in the plasma membrane, typically at the presynaptic ending, whereas synaptobrevin 2 is an intrinsic vesicle protein. We found that syntaxin 3-ir is punctate and is distributed throughout the perikaryon of a DAergic neuron, extending to the cell surface (Fig. 4a–c). Additionally, syntaxin 3-ir is observed within DAergic dendrites in the SNr (Fig. 4d). A similar distribution of SNAP 25-ir within TH-ir somata is shown in Fig. 4 (e–g) and within DAergic dendrites in SNr (Fig. 4h). The presence of synaptobrevin 2-ir within a DAergic perikaryon is illustrated in Fig. 4i–k and in a DAergic dendrite in (Fig. 4l). The observation that within SNr, synaptobrevin 2-ir is much denser in the space surrounding the DAergic profiles than in SNc, results in some chance overlap of synaptobrevin 2-ir with TH-ir processes. Nevertheless, as illustrated in Fig. 4l, there is a low density of synaptobrevin 2-ir puncta consistently associated with the DAergic dendritic profile. Thus our data clearly indicate that both SN DAergic somata and dendrites have a subset of DA release proteins required for exocytosis of synaptic vesicles or some comparable intracellular membrane-bound storage compartment.

Discussion

Our examination of DAergic neurons of the SNc resulted in two main experimental findings. First, two proteins responsible for packaging DA into exocytotic compartments, VMAT2 and v-ATPase, are confined to the DAergic perikaryon and the most proximal portion of its dendrites, but are absent in the more distal regions of the dendrites. The second main finding is that although SNc DAergic somata manifest a subset of proteins generally associated with vesicular release, except for SNAP-25, this group of DA release proteins differs from the subset commonly encountered in presynaptic terminals of fast synapses utilizing amino acid transmitters such as glutamate (reviewed in Südhof, 2004).

A. Proteins involved in DA release by SN DAergic neurons

Prior studies have established that a protein subset consisting of syntaxin, SNAP and synaptobrevin isoforms is required for vesicle fusion (Söllner et al., 1993). Our data reveal a difference in the particular isoforms utilized by the SN DAergic somatodendritic compartment compared to fast synapses mediated by amino acid neurotransmitters. At fast synapses, a commonly encountered group is syntaxin 1a or 1b, SNAP-25 and synaptobrevin 1 or 2 (Söllner et al., 1993; Südhof, 2004). In cells for which neurotransmitter release is relatively slow, other pairings have been identified. For example, at photoreceptor ribbon synapses, Curtis et al. (2008) identified syntaxin 3b, SNAP-25 and synaptobrevin 2, the same subset we found in SN DAergic somata and dendrites. Curtis et al. (2008) also demonstrated that populations of vesicles artificially loaded with either SNAP-25/syntaxin 3b or synaptobrevin 2 bound to each other, indicating their probable association in vivo. Although syntaxin 3 is relatively less abundant than syntaxin 1 in mammalian brain, cDNA clones for all four syntaxin 3 isoforms have been found in mouse brain (Ibaraki et al., 1995).

An analogous situation occurs for isomers of the putative Ca^{2+}-sensing protein, synaptotagmin, an intrinsic protein of the synaptic vesicle. Synaptotagmin 1 and 2 are involved in neurotransmitter release at fast synapses (Xu et al., 2007), but we found them to be absent in SN DAergic perikarya and dendrites. This implies that DAergic cells employ different isomers for somatodendritic release. Indeed, recent evidence suggests that synaptotagmin 7 and possibly synaptotagmin 4 are involved in somatodendritic DA release from mesencephalic cultures (Mendez et al., 2008). Moreover, synaptotagmin 7 is involved in slow exocytosis from chromaffin cells (Schonn et al., 2008). Collectively these findings provide evidence that the molecular components mediating DA release from DAergic somata are unique.

Botulinum toxin A, which cleaves SNAP-25, impairs DA release from SN DAergic perikarya (Bergquist et al., 2002; Fortin et al., 2006), demonstrating a functional role for SNAP-25 (Fig. 4e–h) and supporting the idea that somatodendritic DA release occurs by exocytosis. Other functional studies demonstrated that DA release from the somatodendritic compartment is abolished when a non-selective Ca^{2+} channel blocker such as Cd^{2+} is added to the bathing solution (Jaffe et al., 1998; Patel et al., 2009), or when intracellular Ca^{2+} is reduced by a fast-acting buffer such as BAPTA-AM (Patel et al., 2009). Thus there is good presumptive evidence that the triad of syntaxin 3/SNAP-25/VAMP 2 participates in the exocytosis of a DA-filled organelle. Fortin et al. (2006) found that botulinum B toxin significantly decreased DA release from cultured DAergic somata, consistent with our finding of abundant VAMP 2-ir in DAergic cell bodies. Conversely, the report that neither tetanus toxin nor botulinum toxin B (which cleave VAMP2) altered DA release in SNr (Bergqvist et al., 2002). accords with the relatively low density of VAMP2 we observed in DAergic dendritic profiles of SNr.

B. Somatodendritic DA Storage

The nature of the exocytotic compartment(s) in DAergic somata and dendrites remains to be established. Nirenberg et al. (1996) failed to find aggregates of synaptic vesicles in SN DAergic perikarya, although they are evident in DAergic terminals within the striatum (Pickel et al., 1996). Moreover, VMAT2 is associated with the smooth endoplasmic reticulum in DAergic somata (Nirenberg et al., 1996). Our finding that SN DAergic perikarya lack many proteins typically associated with synaptic vesicles, e.g., sv2a and sv2b, tends to support the idea that DAergic neurons utilize some unconventional exocytotic organelle.

Although there are reports of DA release from SNr (Nieoullon et al., 1977; Rice et al., 1994; Heeringa and Abercrombie, 1995) the mechanism of DA release from DAergic dendrites, whether within SNc or SNr, remains to be clarified. Wilson et al., 1977 reported that synaptic vesicle clusters were found within monoaminergic dendrites, based on their uptake of the false neurotransmitter, 5-hydroxydopamine. However, Wassef et al. (1981) found that only 0.2% of identified DAergic dendrites in SNr had synaptic vesicles, concluding that the processes described previously may not have been DAergic. Groves and Linder (1983), moreover, reported that dendrodendritic contacts between DAergic neurons were absent in SNr. Our data, particularly the absence of sv2a and sv2b in nigral DAergic somata and dendrites, are in agreement with the earlier study by Nirenberg et al. (1996), indicating that clusters of conventional small agranular vesicles are absent.

Whatever the nature of the exocytosing organelle, it must be filled with DA through a combined action of VMAT2 and v-ATPase. The apparent absence of VMAT2 and v-ATPase in distal DAergic dendrites, despite the presence of the SNARE proteins, syntaxin 3b, SNAP-25 and synaptobrevin 2, may indicate differences in the release process between DAergic somata and dendrites. Indeed, at least one form of syntaxin (1a) has been shown to associate with the DA transporter and promote DA release by amphetamine (Binda et al., 2008). Moreover there is as yet no description of putative vesicle exocytosis in distal DAergic dendrites, nor is it known whether or through what mechanism the DAergic exocytotic organelle is recaptured and recycled in the DAergic somatodendritic compartment. The data of the present report are in agreement with a study of retinal DAergic neurons (Witkovsky et al., 2004) in which it was found that VMAT2-ir was confined to the perikaryal region of the soma and the axon terminals, but was absent in dendrites.

Clearly much remains to be determined about the process of DA release from the somatodendritic compartment. Our data suggest that it may be a relatively slow process, consistent with the finding that repeated stimulation of DAergic neurons evokes a progressively diminishing DA release, as assessed by voltammetry (Rice et al., 1997).

Conclusions

Our data provide evidence that the molecular and cellular organization underlying DA release from SN DAergic somata differs substantially from the organization of presynaptic terminals at nerve endings utilizing amino acid neurotransmitters. Additionally our data suggest that DA release from DAergic dendrites in SNr may differ from that seen in the somata, i.e., the somatodendritic compartment may not be homogeneous with regard to the mechanism of DA release. The fate of the DA organelle subsequent to exocytosis is unknown – whether the organelle membrane fuses with the plasma membrane, is recaptured and the site and mechanism of refilling are all important topics for future study, since they will influence strongly the kinetics of dopamine release and recycling in the midbrain.

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Abbreviations

ATPase	adenosine triphosphatase
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’’- tetraacetic acid tetrakis (acetoxymethyl ester)
Ca^{2+}	calcium ion
Cd^{2+}	cadmium ion
CNS	central nervous system
DA	dopamine
DAergic	dopaminergic
g	gram
GABA	gamma-amino butyric acid
ir	immunoreactivity
kg	kilogram
mg	milligram
μm	micrometer
NaCl	sodium chloride
PBS	phosphate-buffered saline
SNAP-25	synaptosome-associated protein-25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulate
sv2a,2b	synaptic vesicle protein 2a,2b
TH	tyrosine hydroxylase
VMAT2	vesicular monoamine transporter type 2
VAMP	vesicle associated membrane protein
w/v	weight per volume

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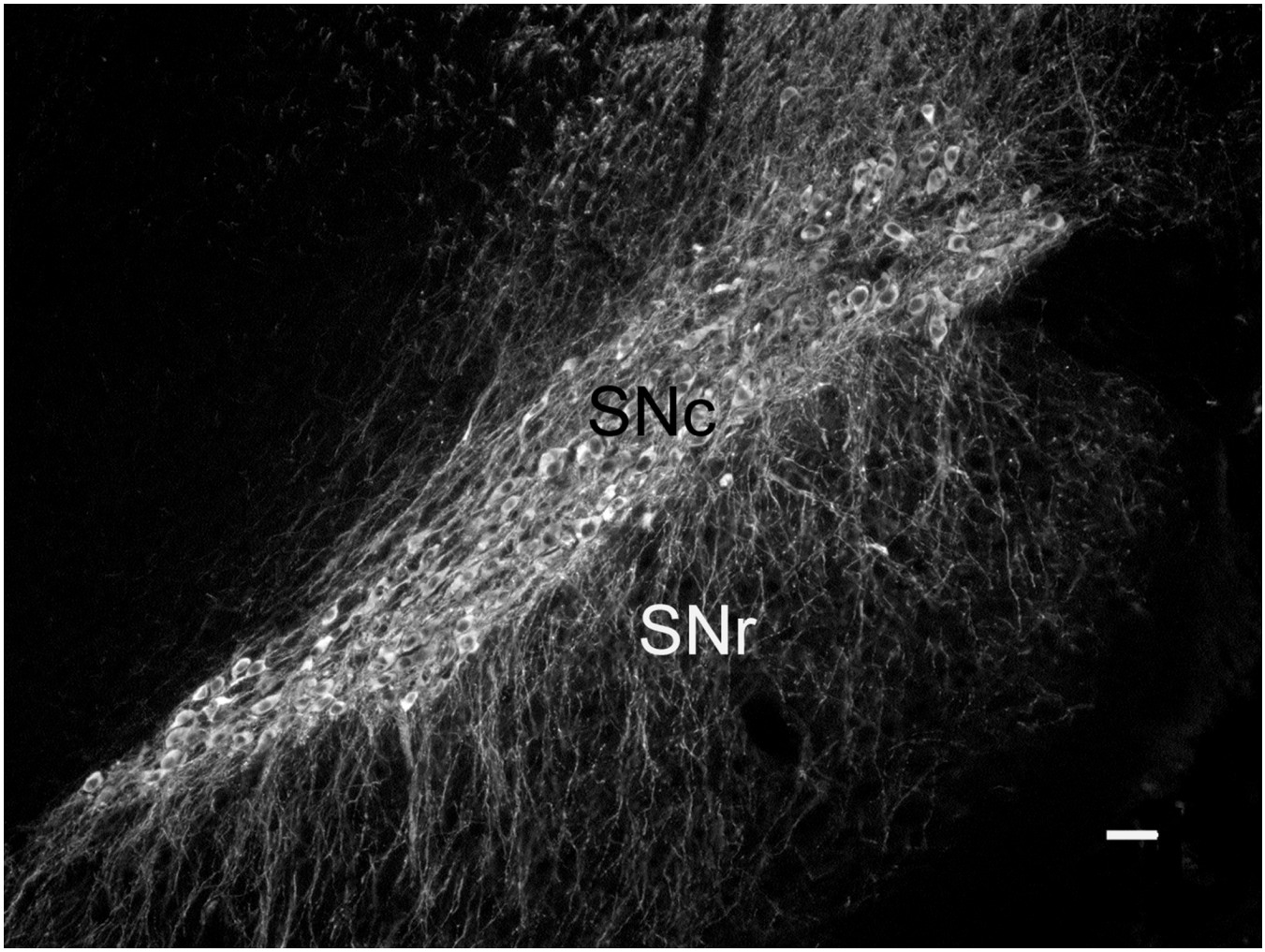

Fig. 1. TH immunostaining in Substantia Nigra

A low magnification view of substantia nigra as revealed by TH-ir. The main subdivisions are pars compacta (SNc) and pars reticulata (SNr). The former consists of a mixture of large perikarya and dendrites, whereas SNr is largely free of DAergic cell bodies but contains abundant TH-ir dendrites with a primarily vertical orientation. Scale bar is 50 μm.

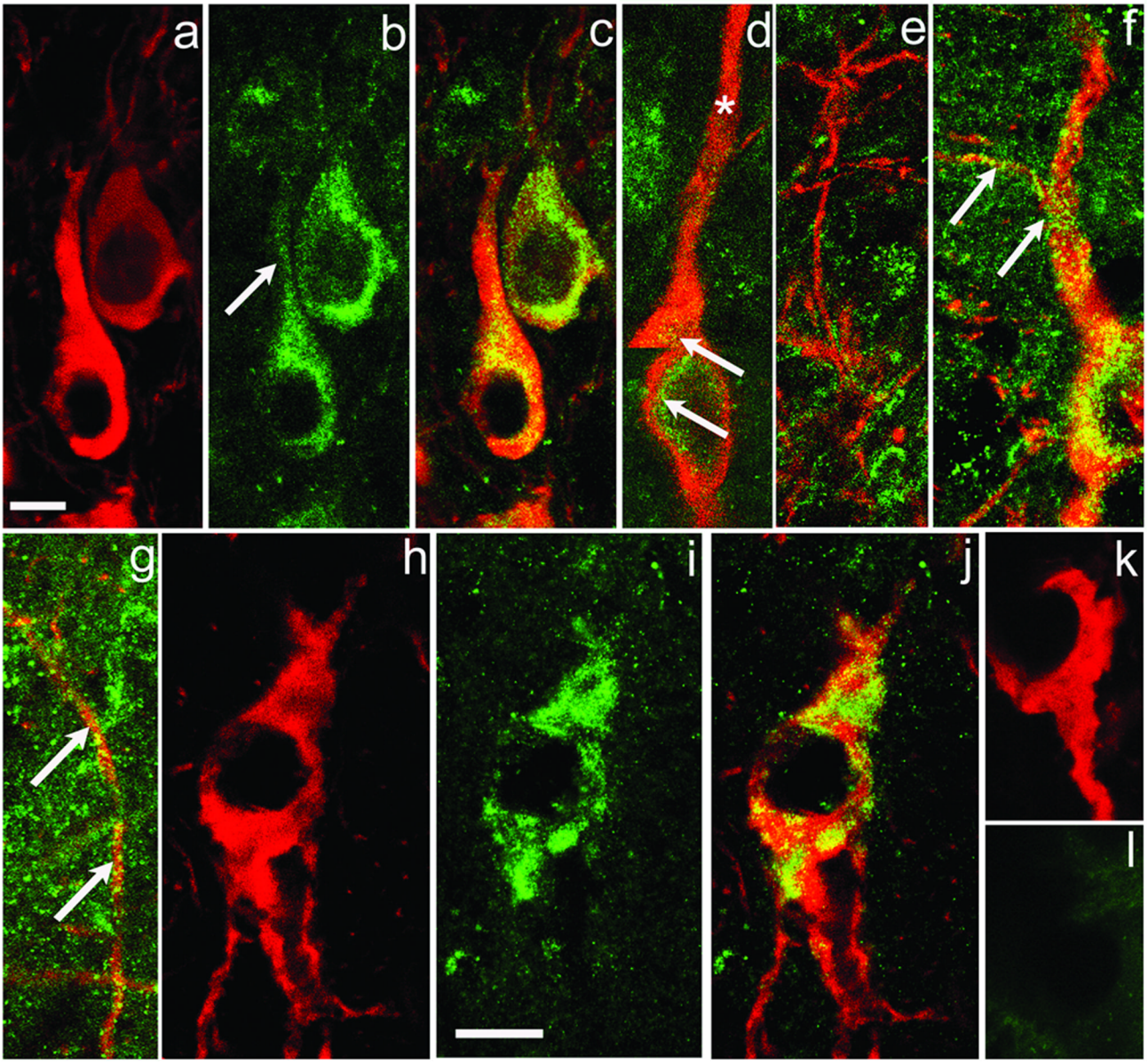

Fig. 2. VMAT2- and v-ATPase-ir colocalize with DAergic perikarya

General Comment: In the color figures, TH-ir is invariably shown in red, the other antibody being tested in green, and yellow indicates colocalization. Panels a,b illustrate, respectively, TH-ir and VMAT2-ir in two DAergic somata. Panel c shows the overlapped images, documenting that VMAT2-ir is located primarily around the DAergic cells' nucleus, extending more sparsely into proximal portions of primary dendrites (b, arrow). Panel d is a montage from a z-stack through a DAergic perikaryon and one primary dendrite. Note abundant VMAT2-ir (yellow green) around the cell's nucleus and initial portion of the dendrite (arrows), but disappearing in the dendrite's more distal portion (*). VMAT2-ir is very sparse in DAergic dendrites within SNr (panel e). However, the dendrite from which the axon branches has abundant VMAT2-ir (arrows, panel f). Panel g shows the continuation of the axon branch (arrows) illustrated in panel f, but turned 90° to fit the figure. Panels h–j illustrate, respectively, TH-ir, v-ATPase-ir and the overlapped images. Similar to VMAT2-ir, v-ATPase-ir is confined almost entirely to the DAergic perikaryon. Panels k and l show, respectively TH-ir and the loss

of v-ATPase-ir following preadsorption of the v-ATPase antibody by its immunogenic peptide. Scale bar in (a) is 10 μm and applies to panels a-g; the scale bar in (i) is 10 μm and applies to panels h-l.

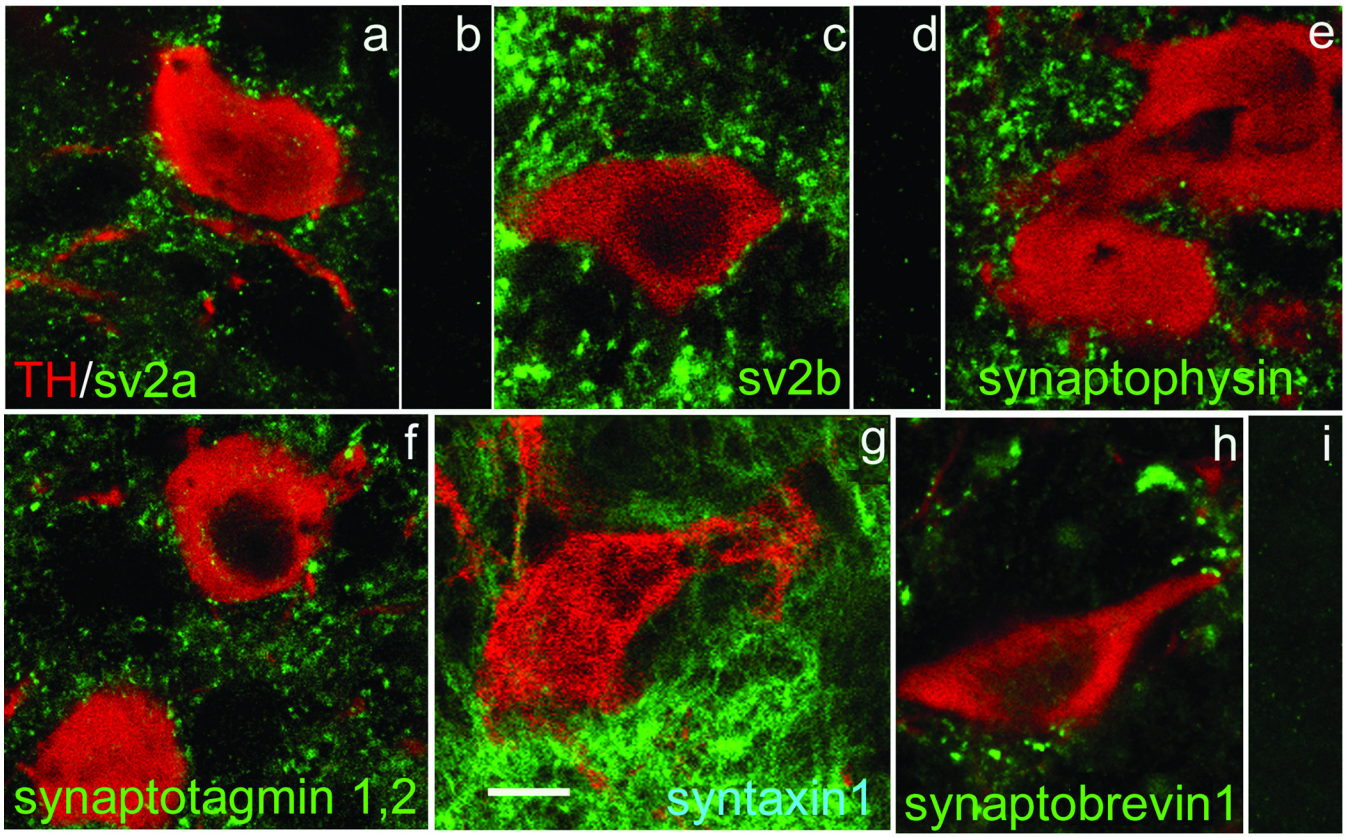

Fig. 3. Presynaptic proteins which do not colocalize with TH in nigral DAergic neurons

Each of panels a,c,e-h shows an overlapped image of TH-ir (red) and the indicated presynaptic protein in green. In every case the presynaptic protein-ir is present in the neuropil and abuts the TH-ir DAergic soma, but is absent within it. Panels b,d, and i show that immunoreactivity was lost for, respectively, sv2a, sv2b and synaptobrevin 1 following preadsorption of the antibody with its immunogenic peptide. Scale bar in (g) is 10 μm and applies to all panels.

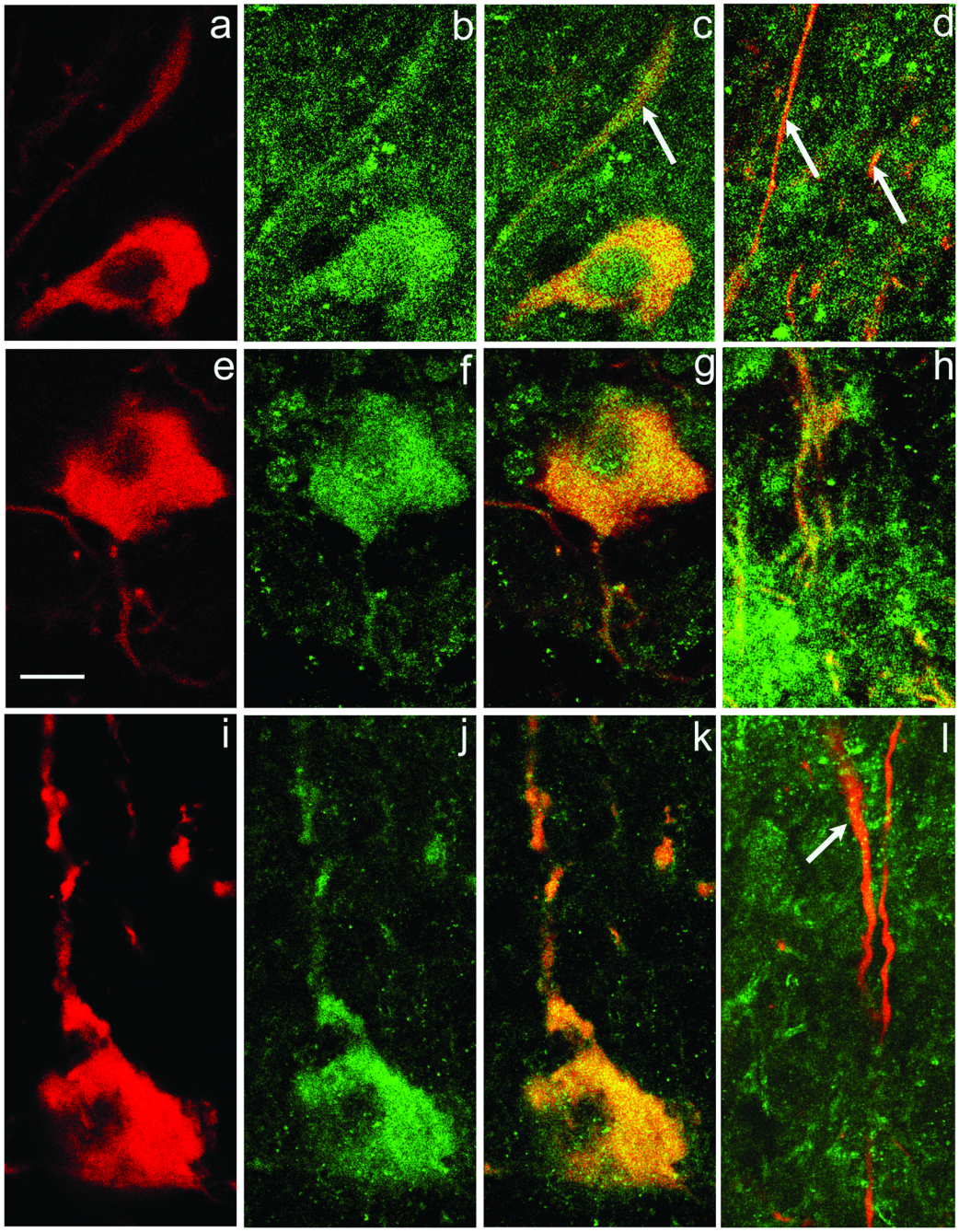

Fig. 4. Colocalization of release proteins with DAergic perikarya and dendrites

Panels a–c illustrate, respectively TH-ir, syntaxin 3-ir and the overlapped image in a DAergic perikaryon. Additionally, colocalization of TH and syntaxin 3-ir is noted in dendrites within SNc (arrow in c) and also in SNr (panel d, arrows). Panels e–h are identically organized to a-d, except that the dopamine release protein is SNAP-25 (green). Panels i–l illustrate the same sequence for TH-ir (red) and synaptobrevin 2 (green). In every case, colocalization is observed both within DAergic perikarya and dendrites, although the density of synaptobrevin 2-ir puncta in dendrites is lower (arrow in l) than that for either SNAP-25 or syntaxin 3-ir puncta. Scale bar in (e) is 10 μm and applies to all panels.

TABLE I

Primary Antibodies

Antibody	Animal	Source	Catalog no.	Dilution
Proton ATPase	Rabbit	Synaptic Systems	111 011	1:500
SNAP-25	Mouse	Synaptic Systems	119 002	1:1000
SV2a	Rabbit	Synaptic Systems	119 002	1:800–2000
SV2b	Rabbit	Synaptic Systems	119–102	1:500–2000
Synaptophysin 1	Mouse	Synaptic Systems	101 011	1:500
Synaptotagmin 1,2	Rabbit	Synaptic Systems	105 002	1:1000
Syntaxin 1	Mouse	Sigma	S0064	1:5000–10000
Syntaxin 3	Rabbit	Abcam	ab4113	1:1000
Synaptobrevin 1	Rabbit	Synaptic Systems	104 002	1:200–1000
Synaptobrevin 2	Rabbit	Synaptic Systems	104 202	1:1000
VMAT2	Rabbit	PhosphoSolutions	2200-VMAT2C	1:3000
TH	Mouse	Chemicon	MAB 318	1:500
TH	Rabbit	Chemicon	AB 152	1:800