Dendritic Projections and Dye-Coupling in Dopaminergic Neurons of the Substantia Nigra Examined in Horizontal Brain Slices From Young Rats

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INTRODUCTION

Dopaminergic neurons of the Substantia Nigra pars compacta (SNC) project to the striatum, where they control neurons involved in the execution of motor functions. The importance of these neurons is highlighted by their involvement in several neurological disorders, including Parkinson’s disease. Although morphological and other properties of SNC neurons have been extensively studied (for review, see Diana and Tepper 2002), two important issues need further attention: the size of their dendritic projection in the rostro-caudal direction and the existence of dye-coupling among these neurons. Previous studies using Golgi staining (Juraska et al. 1977; Phelps et al. 1983) or intracellular labeling in vivo (Preston et al. 1981; Tepper et al. 1987) and in vitro (Häusser et al. 1995; Kita et al. 1986; Nedergaard and Greenfield 1992; Nedergaard and Hounsgaard 1996) have reported the dendritic morphology of these neurons mostly from transverse sections, which disclose medio-lateral and dorso-ventral projections. One study that attempted to assess the rostro-caudal span of dendrites from transverse sections concluded that such projections are very limited (Grace and Bunney 1995). The first objective of our study was to determine the extent of rostro-caudally projecting dendrites of SNC neurons using thick horizontal slices. The second goal was to reassess the existence of dye-coupling between these neurons. Dye-coupling between SNC neurons was described in a single in vivo study published 20 years ago (Grace and Bunney 1983), but has not been reported subsequently. Confirmation of such coupling, usually indicative of communication through gap junctions, would have implications for our understanding of molecular transport and electrical connectivity among these cells.

METHODS

In rats, the SNC is relatively poorly defined in horizontal sections (except parts immediately adjacent to the medial terminal nucleus of the accessory optic tract). To aid positioning of recording electrodes, SNC neurons were retrogradely labeled with Fluoro-Gold (FG). The tracer was injected stereotaxically into the dorsal striatum (0.4%, 0.5 µl each side) of anesthetized P3–P18 Wistar rats. Two or 3 days later, horizontal midbrain slices (400 µm) were cut with a vibratome. Whole cell patch-clamp recordings (EPC-7, List Electronic) were made under infra-red differential interference contrast microscopy (IR-DIC) using microscope (E600FN, Nikon) equipped with epifluorescence and UV-2A, B2A, and G-2A filter blocks. The patch pipette (3–4 MΩ) contained (in mM) 120 K-glucunate, 5 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 1 EGTA, 2 ATP, 0.2 GTP, and 10 glucose (pH 7.3). A thin tube (<100 µm diam), inserted near the tip of the patch pipette, was used to add 7 µl of pipette solution containing a mixture of 2% neurobiotin (NB) and 0.1% lucifer yellow (LY). The dyes were added to the pipette after establishing the whole cell recording configuration, and up to four neurons per slice were filled under direct microscopic control of LY diffusion. This approach prevented leakage of dyes from the pipette prior to establishing a gigaseal (Fig. 2, A1–A4). To validate the capacity of this technique to show dye-coupling, a similar procedure was used to fill hippocampal dentate gyrus cells that are known to show a relatively high degree of dye-coupling during early postnatal stages (Haring et al. 1997).

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RESULTS

Fifty-four dopaminergic SNc neurons were filled with a mixture of LY and NB during whole cell patch-clamp recording in midbrain horizontal slices. All filled neurons met the following criteria: 1) retrograde labeling following striatal injection of FG (Figs. 1, A and B, and 2A2); 2) a hyperpolarization-induced time-dependent inward current \( (I_h) \) under voltage clamp or a depolarizing “sag” in current clamp (both are characteristic of dopaminergic neurons; e.g., Washio et al. 1999) (Fig. 1, D and E); and 3) in current-clamp mode, depolarizing steps caused firing of repetitive action potentials, with a frequency generally not exceeding 10 Hz (Fig. 1E). In addition, immunocytochemical experiments demonstrated that over 90% of FG-labeled neurons were also TH immunoreactive (Fig. 1, B and C).

Diffusion of LY into patched neurons, visualized under direct microscopic control of LY and FG fluorescence during whole cell recording, never resulted in labeling of more than one cell (Fig. 2A4). In addition, no evidence for dye-coupling was found when fixed sections were examined for LY fluorescence or after permanent labeling of neurons using the histochemical protocol for NB detection \( (n = 35; \text{Figs. 2C and 3A}) \). Labeling of additional 19 SNc neurons was made in slices incubated with forskolin (20 or 50 \( \mu \text{M}; \text{n} = 10 \) ) or trimethylamine (10 \( \text{mM}; \text{n} = 9 \) ), agents known to act as gap junction “openers” in many other cell systems (Gladwell and Jefferys 2001; Halliwell and Horne 1998; Kessler et al. 1984; Köhling et al. 2001; Perez-Velazquez et al. 1994). No LY or NB coupling was found in these experiments (Fig. 2B).

To exclude the possibility that these negative findings were...
due to the technique used to fill cells, we used an identical technique to fill neurons in the hippocampal dentate gyrus. Previous studies have demonstrated dye-coupling among dentate gyrus cells filled with LY or NB, particularly at the early postnatal stages of development (Haring et al. 1997; MacVicar and Dudek 1982). Filling of these cells (n = 34; postnatal age: P4–P10) revealed dye-coupling of four filled neurons (12%) under fluorescence (LY labeling; Fig. 4B) or with bright-field microscopy after the avidin-biotin and DAB reactions (NB labeling; Fig. 4C).

Five FG-labeled SNc neurons were also filled with LY alone using sharp intracellular microelectrodes (without exposure to forskolin or trimethylamine). The membrane potential (–63.4 ± 4.0 mV) and firing frequency (1.0–3.5 Hz) were similar to those previously reported for SNc neurons in slices obtained from young rats (e.g., Washio et al. 1999). Although the $I_h$ current was not identified in these neurons, all showed a depolarizing sag during membrane hyperpolarization (data not shown). No evidence for dye-coupling was found in this group, either immediately after cell filling in live slices (Fig. 2B) or on examination of fixed slices.

Dendritic trajectories were examined after camera lucida reconstruction of 54 SNc neurons permanently labeled using the histochemical protocol for NB detection (Fig. 3E). In agreement with previously published data (Häusser et al. 1995; Preston et al. 1981; Tepper et al. 1987), all showed large medio-lateral dendritic projections (529 ± 315 μm lateral and 423 ± 272 μm medial to the cell body). In addition, almost all (53/54) displayed a large rostro-caudal dendritic span (641 ± 226 μm), with particularly prominent caudally projecting dendrites extending 382 ± 212 μm from the cell body (n = 54). The rostro-caudally projecting dendrites examined under high magnification exhibited a predominantly smooth surface. Some dendrites were varicose, particularly in the most distal segments (Fig. 3, C and D).

The expression of mRNA for Cx36 was examined in the ventral midbrain and in individual SNc neurons in an attempt to correlate the absence of dye-coupling between SNc neurons with the message coding for this neuronal connexin. While ventral midbrain tissue tested positively for both Cx36 and TH mRNA (Fig. 4A, lanes 1 and 2), at the single cell level Cx36 expression could not be detected in any of the SNc neurons that tested positive for TH expression (n = 12; Fig. 4A, lanes 3 and 4).

**DISCUSSION**

A novel finding of this study is that SNc neurons have a prominent rostro-caudal dendritic spread. This contrasts with
previous studies, which have analyzed the morphology of such neurons in transverse sections and have identified dendritic projections mainly in the medio-lateral and ventral directions (Häusser et al. 1995; Preston et al. 1981; Tepper et al. 1987), and a single report that dendrites of SNc neurons extend in the rostro-caudal direction only 100–200 μm from the cell body (Grace and Bunney 1995). Identification of rostro-caudal dendrites of SNc neurons is important for several reasons. First, such dendrites, in addition to those projecting in other planes, may play a role in processing excitatory and inhibitory synaptic inputs from a number of local or distant neuronal groups, including the pedunculopontine tegmental nucleus, other parts of the basal ganglia (e.g., subthalamic nucleus), or the cerebral cortex (Fallon and Loughlin 1995). The topographic distribution and dendritic preferences of such inputs require further study. Second, like dendrites projecting in the medio-lateral and ventral directions, rostro-caudally projecting dendrites are likely to be a source of dopamine release. This is supported by our observation that distal parts of such dendrites are often varicose. The significance of local dendritic release of dopamine is not fully understood, but it may play a role in modulating the excitability of SNc neurons (Cheramy et al. 1981; Cragg and Greenfield 1997; Falkenburger et al. 2001). Finally, the existence of such dendrites is of importance for the interpretation of results of a large number of electrophysiological and other studies conducted on SNc neurons in horizontal or parasagittal, rather than conventional transverse, midbrain slices (e.g., Falkenburger et al. 2001; Grillner et al. 2000; Marinelli et al. 2001). In such studies, the ventrally and dorsally (in horizontal sections) or medially and laterally (in parasagittal sections) projecting dendrites are truncated, and only those extending in other planes (including the rostro-caudal direction) would determine the properties of these neurons.

Our other main observation was the absence of dye-coupling in SNc neurons examined in brain slices. This negative finding is in contrast to the in vivo study performed on adult rats by Grace and Bunney (1983), who reported that injections of LY into single SNc neurons with intracellular microelectrodes often resulted in labeling of two to five neurons. In our experiments, several measures were used to facilitate dye-coupling, but despite such efforts, we could not identify its presence. First, following the previous in vivo study that found a particularly high incidence of dye-coupling between neurons oriented rostro-caudally to each other and the presence of colabeled neurons in consecutive transverse sections (Grace and Bunney 1983), we filled SNc neurons in horizontal slices, thus preserving their spatial rostro-caudal relationship. Second, our experiments were performed on slices obtained from young animals, since other studies have established that dye (and electrotonic) coupling, as well as expression of relevant connexins, generally peak during the initial postnatal period (Belluardo et al. 2000; Rörg et al. 1995; Söhl et al. 1998). Third, both LY and NB were used in most of our recordings, as previous studies of dye-coupled cells in other parts of the CNS have shown that NB (323 Da, positively charged) can penetrate gap junctions more effectively than LY (435 Da, negatively charged) (e.g., Vaney 1991). Fourth, some of our cells were labeled during exposure of slices to the potential gap junction “openers,” forskolin and trimethylamine. Forskolin, a potent adenylyl cyclase activator that can stimulate connexin phosphorylation through a cAMP-dependent pathway (Dowling-Warriner and Troscu 2000), has been shown to increase gap junction coupling among CA3 pyramidal neurons in the hippocampus (Gladwell and Jefferys 2001) and sympathetic neurons (Kessler et al. 1984). Trimethylamine, which acts through intracellular alkalinization, increased communication through pH-sensitive gap junctions in CA1 and CA3 pyramidal cells of the hippocampus (Köhling et al. 2001; Perez-Velazquez et al. 1994) and in Island of Calleja granule cells (Halliwell and Horne 1998). Fifth, slices were maintained at 34°C, since lower temperatures have been shown to reduce the opening state of gap junctions (Chen and DeHaan 1993). Finally, to address the possibility that neurons dialyze and lose cytosolic components that control gap junctions during whole cell recording, we also used sharp intracellular microelectrodes with similarly negative results. Dye-coupling has, however, been identified in other neural systems with recording and labeling performed with patch pipettes in slices obtained from postnatal animals (Connors et al. 1984; Kim et al. 1995; Rekling and Feldman 1997). In addition, our control experiments using patch pipettes confirmed the presence of dye-coupling between hippocampal dentate gyrus neurons (Haring et al. 1997; MacVicar and Dudek 1982).

It has been reported that the mRNA message for Cx36 (neuronal connexin) is expressed in the SNc region of the ventral midbrain (Condorelli et al. 2000). However, this connexin is widely distributed in the CNS (Condorelli et al. 2000; Parenti et al. 2000; Rash et al. 2000), and therefore its expression cannot be taken as evidence of interneuronal communication through gap junctions. Our own RT-PCR analysis confirmed the expression of this connexin in tissue dissected from the ventral midbrain, but not in 12 single SNc neurons. It should also be noted that a recent study by Belluardo et al. (2000) did not find immunolabeling for Cx36 in the substantia nigra either in the adult or the developing rat brain.

In summary this study has identified, for the first time, extensive dendritic projections of dopaminergic SNc neurons in the rostro-caudal direction. In addition, using strict precautions to prevent leakage of NB and LY from patch pipettes, no dye-coupling could be identified between these neurons. The negative finding is in agreement with the study by Walsh et al. (1991), who observed dye-coupling between SNc neurons in brain slices obtained from cat fetuses but not at postnatal stages. The difference between these in vitro results and the multiple labeling (as well as indirect evidence for electrical coupling) reported by Grace and Bunney (1983) might be due to neuronal damage or extracellular leakage from sharp microelectrodes and uptake of dye by adjacent cells, which can occur when filling cells in vivo. Alternatively, it could be due to other differences in experimental conditions associated with in vitro versus in vivo recording or to the development of gap-junction communication between SNc neurons after P20. Further studies with double recording from pairs of SNc neurons are needed to establish whether these neurons are electrically coupled in slices and whether there is a dissociation between dye and electrical coupling in postnatal neurons examined in vitro, similar to that previously described in neocortical slices (Gibson et al. 1999).

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