Morphological and Physiological Properties of Parvalbumin- and Calretinin-Containing γ-Aminobutyric Acidergic Neurons in the Substantia Nigra

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ABSTRACT

Evidence for the existence of different populations of γ-aminobutyric acid (GABA)-ergic neurons in the substantia nigra comes partially from anatomical studies, which have shown there to be little if any overlap between the calcium-binding proteins parvalbumin and calretinin in individual neurons, suggesting that these may represent neuronal subtypes with distinct electrophysiological and/or anatomical properties. We obtained whole-cell recordings from neurons in the substantia nigra pars reticulata in rat brain slices and labeled them with biocytin, followed by immunocytochemical staining for parvalbumin and calretinin. In other cases, neurons were retrogradely labeled from the thalamus or tectum and immunocytochemically identified to determine their projection sites. Intracellularly stained neurons were found to have a variety of somatic sizes and shapes. Reconstructions revealed that all parvalbumin- and calretinin-positive neurons issued at least one axon collateral, which ramified within the substantia nigra pars reticulata and/or pars compacta. Local collaterals were of medium caliber and branched modestly, expressing many long, smooth segments that then issued numerous en passant or terminal boutons, consistent with previous in vivo studies. There were no clear differences in the electrophysiological or morphological properties of neurons expressing parvalbumin or calretinin. Retrograde tracing experiments revealed that both parvalbumin- and calretinin-containing neurons project nonpreferentially to the thalamus or tectum. In sum, the parvalbumin- and calretinin-containing GABAergic neurons of the substantia nigra pars reticulata cannot be differentiated on the basis of their electrophysiological properties, morphological properties, or target nuclei, and both parvalbumin- and calretinin-containing projection neurons issue local axon collaterals that arborize within the substantia nigra. J. Comp. Neurol. 500: 958–972, 2007. © 2006 Wiley-Liss, Inc.

Indexing terms: basal ganglia; calcium-binding proteins; immunocytochemistry; whole-cell recording; axon collaterals; pars reticulata

Our knowledge of the morphological and physiological properties of γ-aminobutyric acid (GABA)-ergic neurons in the substantia nigra lags behind that of the constituent neurons of other basal ganglia nuclei, such as the neostriatum (Tepper and Bolam, 2004) and globus pallidus (Kita and Kitai, 1991, 1994; Kita, 1994; Nambu and Llinas, 1994, 1997; Cooper and Stanford, 2000, 2002; Kita and Kita, 2001), where the presence of distinct neuronal subtypes has been described. Perhaps the most complete description of GABAergic neuronal subtypes in a basal ganglia nucleus has been obtained in the neostriatum, where three classes of GABAergic interneurons have been shown to be morphologically and physiologically distinct, as well

as to contain calcium-binding proteins different both from each other and from the GABAergic projection neurons of that nucleus (Bennett and Bolam, 1993; Kawaguchi, 1993; Kubota et al., 1993; Kawaguchi et al., 1995; Figueredo-Cardenas, 1996; Rymar et al., 2004).

In the substantia nigra, both in vivo and in vitro physiological studies of neurons have largely emphasized the differences that exist between dopaminergic and GABAergic neurons, with little study into the potential differences among the GABAergic neurons themselves (Wilson et al., 1977; Deniau et al., 1978a; Guyenet and Aghajanian, 1978; Nakanishi et al., 1987; Yelnik et al., 1987; Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991; Richards et al., 1997; Gulacsi et al., 2003). The GABAergic projection neurons of the substantia nigra pars reticulata (SNr) have two primary targets, the thalamus and superior colliculus (Rinvik, 1975; Clavier et al., 1976; Faull and Mehler, 1978; Guyenet and Aghajanian, 1978; Tokuno and Nakamura, 1987; Harting et al., 1988; Kemel et al., 1988; Williams and Faull, 1988; Bickford and Hall, 1992; Deniau and Chevalier, 1992; Redgrave et al., 1992; Mana and Chevalier, 2001; Sidibe et al., 2002), and their function is to inhibit the principal neurons in those nuclei (Ueki et al., 1977; Deniau et al., 1978b; MacLeod et al., 1980; Chevalier et al., 1981a,b, 1985; Ueki, 1983; Deniau and Chevalier, 1985; Karabelas and Moschovakis, 1985; Pare et al., 1990). In addition to the inhibitory effects that nigral GABAergic neurons have on targets outside the basal ganglia, electrophysiological and morphological studies have shown that the dopaminergic neurons of the substantia nigra receive a strong inhibitory influence from GABAergic neurons in the same nucleus (MacNeil et al., 1978; Walters and Lakoski, 1978; Grace and Bunney, 1979, 1985a,b; Waszczak et al., 1980; Hajos and Greenfield, 1994; Hausser and Yung, 1994; Tepper et al., 1995, 2002; Celada et al., 1999, Paladini et al., 1999; Gulacsi et al., 2003; Lee et al., 2004; Saitoh et al., 2004). As with the dopaminergic neurons of the substantia nigra, nigral GABAergic neurons also receive inhibitory input from other nigral GABAergic neurons; antidromic stimulation of GABAergic projection neurons from either the thalamus or the superior colliculus produces monosynaptic inhibitory postsynaptic potentials (IPSPs) in nigral GABAergic neurons (Deniau et al., 1982). As would be expected in light of the physiological data demonstrating the intranigral inhibitory actions of GABAergic projection neurons, these neurons have been shown to issue axon collaterals that arborize extensively throughout both substantia nigra pars compacta and pars reticulata (Deniau et al., 1982; Grofova et al., 1982; Kemel et al., 1988; Nitsch and Riesenberg, 1988; Tepper et al., 2002; Mailly et al., 2003).

Although it is clear that local GABAergic signaling within the substantia nigra exists and is carried out, at least in part, through axon collaterals from projection neurons of the same nucleus, it has been suggested that locally projecting interneurons exist and also contribute to intranigral signaling (Juraska et al., 1977; Francois et al., 1979; Grace and Bunney, 1979, 1985a,b; Lacey et al., 1989; Johnson and North, 1992; Bontempi and Sharp, 1997; Hebb and Robertson, 2000). Further evidence for heterogeneity among nigral GABAergic neurons comes from anatomical studies showing that subsets of these neurons express the calcium-binding proteins calretinin and parvalbumin. These calcium-binding proteins are only rarely colocalized in single neurons (Gonzalez-Hernandez and Rodriguez, 2000). The difficulty in staining for glutamic acid decarboxylase, the synthetic enzyme for GABA, without the use of colchicine makes quantification of parvalbumin- and calretinin-containing neurons difficult, but estimates have suggested that more than 80% of glutamic acid decarboxylase-containing neurons also contain parvalbumin, whereas a slightly lower percentage ($\sim$60%) contains calretinin (Gonzalez-Hernandez and Rodriguez, 2000). Additionally, there may be spatial differences in the distribution of the calcium-binding proteins among nigral GABAergic neurons, insofar as the rostromedial portion of the pars reticulata is largely devoid of parvalbumin-containing neurons and instead comprises primarily calretinin-containing neurons (Celio, 1990; Hontanilla et al., 1997; Gonzalez-Hernandez and Rodriguez, 2000).

Thus, it is possible that calretinin and parvalbumin are expressed in neuronal subtypes with distinct physiological and/or morphological properties or differences in their projection sites. The calcium-binding protein phenotypes of the neurons issuing axon collaterals within the substantia nigra are unknown. In addition, at least some nigrocollicular and nigrothalamic projection neurons express parvalbumin immunoreactivity (Rajakumar et al., 1994), but the targets of calretinin-containing, GABAergic neurons in the substantia nigra are also unknown.

The present experiments focused on examining the physiological and morphological properties of immunocytochemically identified GABAergic neurons in the substantia nigra to determine whether neurons expressing different calcium-binding proteins are also physiologically and/or morphologically distinct. To this end, we performed whole-cell recordings from electrophysiologically identified nondopaminergic SNr neurons in rat brain slices and labeled them with biocytin. The calcium-binding protein phenotype of the recorded neurons was assessed by using immunocytochemistry for parvalbumin and calretinin, followed by embedding and reconstruction to study their morphological properties. Additionally, the projection sites of parvalbumin- and calretinin-positive neurons were studied by using retrograde labeling from the superior colliculus and thalamus, followed by immunocytochemistry for parvalbumin or calretinin.

MATERIALS AND METHODS
Whole-cell recording

In vitro visualized whole-cell current clamp recordings of nigral GABAergic neurons were obtained from 300-μm coronal slices of young adult male Sprague Dawley rat brain (21–28 days, n = 20). All procedures were performed with the approval of the Rutgers University Institutional Animal Care and Use Committee and in accordance with the NIH *Guide to the care and use of laboratory animals*. Animals were deeply anesthetized with 150 mg/kg ketamine and 30 mg/kg xylazine i.p. and transcardially perfused with ice-cold, modified Ringer's solution containing 225 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 28 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 7 mM glucose, 1 mM ascorbate, and 3 mM pyruvate that was bubbled with 95% O_2 and 5% CO_2 (pH 7.3) until the blood had been replaced with this solution. The brain was then quickly removed and trimmed to a block containing the

The Journal of Comparative Neurology. DOI 10.1002/cne

960   C.R. LEE AND J.M. TEPPER

midbrain. Sections were cut in the same medium with a Vibroslice (World Precision Instruments, Sarasota, FL; model NVSL) and were immediately transferred to normal Ringer's solution containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose, 1 mM ascorbate, 3 mM pyruvate, and 0.4 mM myoinositol, which was heated to 34°C and continuously bubbled with 95% O_2 and 5% CO_2 (pH 7.3) for 1 hour prior to recording and then maintained at room temperature until use. Slices were transferred to the recording chamber and submerged in continuously flowing buffer, which was heated to approximately 34°C. Neurons were visualized with a ×40 water-immersion objective by using an Olympus BX50WI fixed-stage microscope with infrared differential interference contrast microscopy and a MTI CCD-300 CCD camera (Dage-MTI, Michigan City, IN).

Recordings were obtained by using borosilicate pipettes pulled from 1.5-mm-o.d. capillary tubing (World Precision Instruments) with a Narishige PP-83 vertical pipette puller (Narishige International USA, Inc., East Meadow, NY). Pipettes were filled with a solution containing 129 mM potassium gluconate, 11 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, 3 mM Na_2-ATP, and 0.3 mM Na_3-GTP (pH 7.2) into which 0.3% biocytin (Sigma-Aldrich Co., St. Louis, MO) was dissolved by sonication. Pipettes had resistances of 4–6 MΩ measured from short current pulses. Recordings were amplified with a Neurodata IR-283 amplifier and sampled at 40 kHz with a Micro1401 (Cambridge Electronic Design, Cambridge, United Kingdom) and a PC running Signal version 3.05 (Cambridge Electronic Design).

Both the substantia nigra pars compacta and pars reticulata consist of dopaminergic neurons and nondopaminergic, presumably GABAergic, neurons, which differ from the dopaminergic neurons in terms of their higher firing rate, narrower spike width, short-latency and small-amplitude afterhyperpolarization, and less pronounced voltage sag in response to hyperpolarizing current pulses (Kita et al., 1986; Nakanishi et al., 1987; Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991; Richards et al., 1997; Gulacsi et al., 2003; Atherton and Bevan, 2005; Ibanez-Sandoval et al., 2006). These criteria were used to differentiate nigral GABAergic neurons from dopaminergic neurons. The data presented here were obtained from neurons that were classified as GABAergic based on these characteristics.

After recording, slices were immediately transferred to chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) where they were stored overnight at 4°C. The slices were then transferred to phosphate-buffered saline (PBS; pH 7.4) prior to fluorescent immunocytochemistry. All efforts were made to minimize recording time (<5 minutes) to reduce dilution of the cell interior with the pipette solution and preserve detection of intracellular antigens.

Electrophysiological data analysis

Data were analyzed in Signal version 3.05 (Cambridge Electronic Design). Action potential data were obtained from four consecutive spontaneous spikes and averaged. The input resistance was measured from single, small-amplitude hyperpolarizing current pulses delivered from rest, which were sufficient to halt spiking. The voltage deflection used in calculating the input resistance was determined to be the difference between the voltage 50 μsec before current onset and the most hyperpolarized voltage obtained during the current pulse. Action potential amplitudes were calculated as the voltage difference between the action potential threshold, which was defined as the time point reaching 15% of the maximal rising slope achieved during the action potential upstroke, and the peak of the action potential. The spontaneous spike width was calculated at half-amplitude between the action potential threshold and the peak of the action potential. The afterhyperpolarization amplitude and trough time were determined by calculating the difference between the threshold and the most negative voltage achieved during the afterhyperpolarization. The spontaneous firing rate was calculated for each neuron from a 1-second sweep of spontaneous activity. Sag amplitudes were calculated as the voltage difference between the most hyperpolarized voltage measured during a 500-msec, 0.2-nA hyperpolarizing current pulse delivered from rest and the voltage 500 μsec prior to current offset. All data are presented as mean ± SEM. Mean values were compared via one-way analysis of variance with the criterion for significance set to $P < 0.05$, followed by Scheffe's multiple-comparison test with α = 0.05. Statistics were computed in SAS version 8.02 (SAS Institute Inc., Cary, NC). Voltages reported have been corrected for the liquid junction potential, which was estimated to be 14.4 mV in JPCalc (Barry, 1994).

Immunocytochemistry

Sections were pretreated with 1% sodium borohydride, followed by 10% methanol and 3% H_2O_2 in phosphate-buffered saline (PBS) prior to incubation in 10% normal goat serum, 2% bovine serum albumin, and 0.5% Triton X-100 for 1 hour without resectioning. Sections were then incubated in a solution containing 1:1,500 rabbit anticalretinin polyclonal antibody (Chemicon International, Temecula, CA; No. AB5054) and 1:1,500 mouse antiparvalbumin monoclonal antibody (Sigma-Aldrich Co.; No. P 3088) along with 10% normal goat serum, 2% bovine serum albumin, and 0.5% Triton X-100 (Sigma-Aldrich Co.) in PBS for 24 hours at room temperature. The anticalretinin antibody was raised against recombinant full-length rat calretinin and recognizes a 32-kDa protein on Western blot (data provided by Chemicon International). The antiparvalbumin antibody was derived from the PARV-19 hybridoma, which was raised against frog muscle parvalbumin and recognizes a single 12-kDa protein on Western blot (data provided by Sigma-Aldrich Co.). The pattern of parvalbumin and calretinin staining observed in the substantia nigra using these antibodies was similar or identical to that previously described (Celio, 1990; Arai et al., 1991; Rajakumar et al., 1994; Alfahel-Kakunda and Silverman, 1997; Hontanilla et al., 1997; Gonzalez-Hernandez and Rodriguez, 2000). The specificity of the aforementioned antibodies for their antigens has been determined by preadsorption with the appropriate purified calcium-binding proteins and thoroughly described by others (Hackney et al., 2005).

After washing three times for 10 minutes each in PBS, sections were transferred to a solution containing 1:300 AMCA-conjugated streptavidin, 1:200 Cy3 goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA), 1:200 Oregon green goat anti-mouse (Molecular Probes, Eugene, OR), 10% normal goat serum, and 2%

The Journal of Comparative Neurology. DOI 10.1002/cne

MORPHOLOGY AND PHYSIOLOGY OF NIGRAL GABAERGIC NEURONS 961

bovine serum albumin in PBS at 4°C overnight. After three 10-minute washes in PBS, sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and observed by using an Olympus BX50WI fluorescent microscope.

For control experiments (n = 4), sections were processed identically with the exception that the primary antibodies against parvalbumin and calretinin were omitted. In these cases, the recorded neuron was identified by biocytin fluorescence, but staining for parvalbumin and calretinin was absent. Fluorescent images were acquired with a Nikon D70 color digital camera, and those presented here were adjusted for brightness and contrast in Adobe Photoshop CS.

After immunocytochemical identification, the sections were washed for 3 × 10 minutes in 0.1 M PB, followed by 10% methanol and 3% H_2O_2 for 15 minutes, and incubated with avidin-biotin-peroxidase complex (Vector Laboratories; 1:200) and 0.1% Triton X-100 overnight at 4°C. After washing for 6 × 10 minutes in 0.1 M PB, the sections were reacted with 3,3′-diaminobenzidine (0.025%) and H_2O_2 (0.0008%) in PB with nickel intensification (2.5 mM nickel ammonium sulfate and 7 mM ammonium chloride). The sections were then postfixed in osmium tetroxide (0.1% in PB) for 30 minutes, dehydrated through a graded series of ethanol followed by propylene oxide, and infiltrated overnight with a mixture of propylene oxide and epoxy resin (Durcupan; Fluka Chemie, Buchs, Switzerland). The sections were then transferred to fresh resin mixture for several hours and finally flat-embedded between glass slides and coverslips.

Neuronal reconstruction and morphometry

The aforementioned diaminobenzidine (DAB) reaction with nickel intensification resulted in a dense, black DAB reaction product throughout the soma as well as the dendritic and axonal arborizations of the recorded neurons allowing for their reconstruction. Because much of the axon and dendritic arborization was frequently lost during sectioning, full neuronal reconstructions were carried out only on neurons where the greatest amount of the axonal and/or dendritic arborizations had been retained.

Somatic measurements were obtained from embedded neurons that had been photographed at ×40–100 with a Leica DMRE microscope and a Hamamatsu Orca-ER digital camera (Hamamatsu Corp., Bridgewater, NJ). Images were captured and analyzed in Openlab version 4.0.2 (Improvision Inc., Boston, MA). The shape factor is equal to $4\pi A/P^2$, where A is the area and P is the perimeter of the measured neuron. A perfect circle has a shape factor of 1.0; irregular shapes have smaller shape factors. Embedded neurons were drawn under a ×60 oil-immersion objective with a Nikon Optiphot microscope fitted with a drawing tube. Sholl analyses were performed on neuronal reconstructions using concentric circles separated by 20 μm (Sholl, 1953).

All data are presented as mean ± SEM. Mean values were compared via one-way analysis of variance or *t*-test where appropriate. In the case of the Sholl analyses, the mean number of intersections at each radius was compared with a two-way analysis of variance using the general linear model with distance as a repeated measure. In all cases, the criterion for significance was set to $P < 0.05$. One-way analyses of variance were followed by Scheffe's multiple comparison test with $\alpha = 0.05$. Statistics were computed in SAS version 8.02 (SAS Institute Inc.).

Retrograde labeling of substantia nigra pars reticulata projection neurons

Retrograde labeling of SNr projection neurons was accomplished through bilateral infusion of fluorogold into either the superior colliculus or the ventral thalamus. Twelve adult male Sprague-Dawley rats weighing between 220 and 280 g were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg). Fluorogold (Fluorochrome, Denver, CO; 4% in 0.9% saline, 1 μl) was then infused over a period of 10 minutes into the superior colliculus (6.8 mm posterior to bregma, 1.0 mm lateral to the midline, 3.0 mm below the cortical surface) or ventral anterior thalamus (5.1 mm anterior to lambda, 2.0 mm lateral to the midline, 6.1 mm below the cortical surface) with a Hamilton syringe (Hamilton Co., Reno, NV).

After 5–7 days, the rats were deeply anesthetized with ketamine (150 mg/kg) and xylazine (30 mg/kg) and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in PB. The brains were harvested and stored in sucrose (30% w/v) dissolved in paraformaldehyde (4%) overnight at 4°C. The brains were then sectioned with a freezing microtome, and 40-μm coronal sections of substantia nigra and 80-μm sections of the injection site were collected.

Sections were pretreated with 1% sodium borohydride, followed by 10% methanol and 3% H_2O_2 in PBS prior to incubation in 10% normal goat serum, 2% bovine serum albumin, and 0.5% Triton X-100 for 1 hour. This was followed by incubation in a solution containing either 1:1,500 rabbit anticalretinin antibody (Chemicon International) or 1:1,000 mouse antiparvalbumin antibody (Sigma-Aldrich Co.), as described earlier, along with 10% normal goat serum, 2% bovine serum albumin, and 0.5% Triton X-100 in PBS for 24 hours at room temperature. After washing for 3 × 10 minutes in PBS, sections were transferred to a solution containing either 1:200 Cy3 goat anti-rabbit IgG or 1:200 Cy3 goat anti-mouse IgG with 10% normal goat serum and 2% bovine serum albumin in PBS at 4°C overnight. After three 10-minute washes in PBS, sections were mounted in Vectashield (Vector Laboratories) and observed in an Olympus BX50WI fluorescent microscope. Fluorescent images were adjusted for brightness and contrast in Adobe Photoshop CS.

RESULTS

Among 55 neurons recorded, 25 were identified as being immunoreactive for parvalbumin and 16 for calretinin, and 13 did not exhibit any apparent immunoreactivity (Fig. 1). One neuron exhibited immunoreactivity for both parvalbumin and calretinin. All of the neurons were located in the SNr.

Morphological properties of immunocytochemically identified neurons

It was impossible to determine whether neurons that exhibited no apparent immunoreactivity for either calcium-binding protein were in fact devoid of calcium-binding proteins or the calcium-binding proteins were not detected using the current methods. Such a failure to detect the calcium-binding proteins could result from di-

The Journal of Comparative Neurology. DOI 10.1002/cne

962   C.R. LEE AND J.M. TEPPER
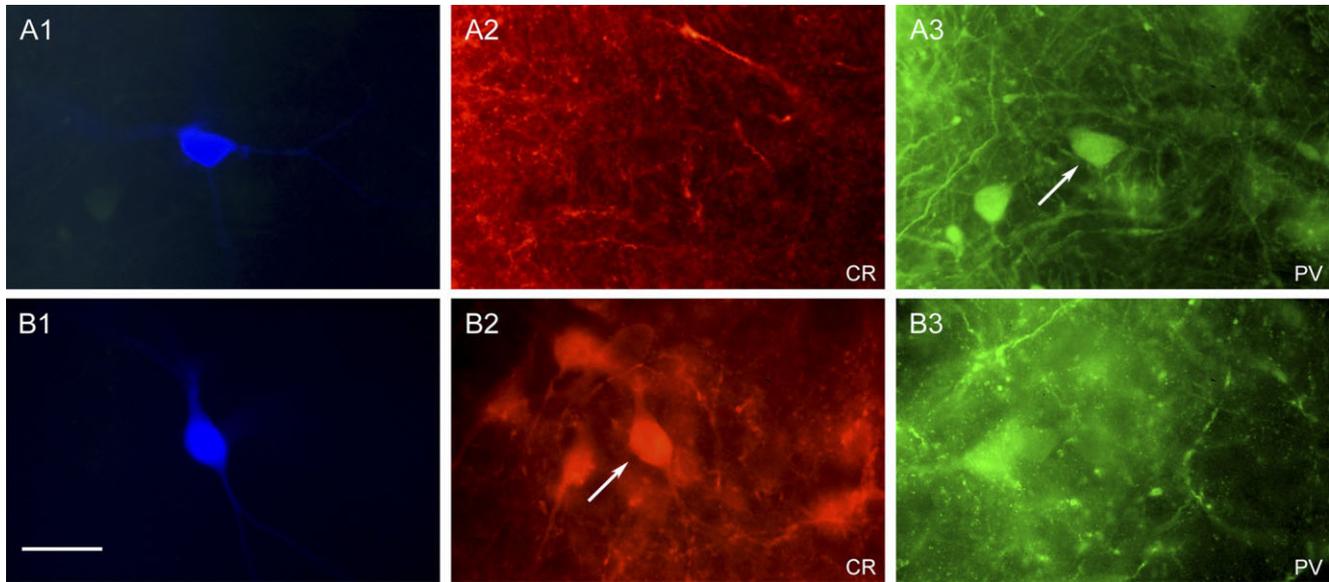
Fig. 1. Immunocytochemical identification of GABAergic neurons in the substantia nigra. **A1:** A recorded neuron filled with biocytin is shown to be devoid of immunoreactivity for calretinin (**A2**) but is immunoreactive for parvalbumin (**A3**, arrow). **B1:** Another neuron is shown to exhibit immunoreactivity for calretinin (**B2**, arrow) but is devoid of immunoreactivity for parvalbumin (**B3**). CR, calretinin; PV, parvalbumin. Scale bar = 25 μm.

lution of the intracellular antigens by the pipette solution during whole-cell recording, despite all efforts to minimize the time spent recording the cells. Therefore, the morphological properties of these neurons will not be presented in detail here. These neurons did not, however, exhibit any obvious morphological differences from the neurons in which calcium-binding proteins were detected (data not shown). One neuron exhibited both parvalbumin and calretinin immunoreactivity and generally shared the same properties as either parvalbumin- or calretinin-immunoreactive neurons, including collateralization of the axon (see below).

The morphological characteristics of the immunocytochemically identified neurons were largely similar regardless of the immunocytochemical identity of the neuron. In all cases, the somatodendritic and axonal properties exhibited by the neurons were consistent with those previously reported in nigral GABAergic projection neurons (Deniau et al., 1982; Grofova et al., 1982; Mailly et al., 2001, 2003). Figures 2 and 3 show reconstructions of representative parvalbumin- and calretinin-containing neurons, respectively.

Morphometric data were obtained from 47 neurons. Most somata were ovoid or fusiform in shape and had shape factors that ranged from 0.41 to 0.85 (mean 0.71 ± 0.01). Neuronal somata ranged from 115.86 μm² to 259.77 μm² in area (mean 172.96 ± 5.61 μm²) and had perimeters ranging from 43.62 μm to 69.72 μm (mean 55.43 ± 0.95 μm). Neurons exhibiting immunoreactivity for the different calcium-binding proteins did not differ in terms of these parameters (Table 1). Because only a single neuron was found to exhibit immunoreactivity for both parvalbumin and calretinin, statistical comparison of the morphometric properties of this neuron (which were in the same range as those for parvalbumin- and calretinin-immunoreactive neurons) with the others was not possible.

Full reconstructions were completed on a total of 21 neurons, 12 of which were identified as containing parvalbumin,

eight which contained calretinin, and one which contained both parvalbumin and calretinin. Neurons issued between two and six primary dendrites (mean 4.0 ± 0.2), which arborized and extended for 303.8 ± 17.6 μm (range 160–500 μm) as estimated by the most distal line crossed by a dendrite in the Sholl analyses. Most of the proximal dendrites were aspiny, but some higher order dendrites possessed spines or longer spine-like appendages (see Fig. 3, top left). The higher order dendrites of all neuronal types were often markedly varicose and sometimes appeared beaded. Overall, dendrites made 59.7 ± 4.4 intersections (range 30–103) in the Sholl analyses. Sholl analyses did not reveal any differences in the number of intersections made by dendrites of parvalbumin- or calretinin-containing neurons as assessed by a two-way analysis of variance using the general linear model with distance as a repeated measure. Dendrites terminated in six to 24 tips (mean 13.9 ± 1.1). There were no apparent differences in the dendritic properties of nigral GABAergic neurons containing parvalbumin or calretinin (Table 1).

In most neurons, regardless of calcium-binding protein phenotype, a single, thick, main axon emerged from the soma, a somatic extension, or a primary dendrite and typically coursed through the substantia nigra, issuing one or more collaterals and ended in a retraction bulb where the main axon had been transected. Axon collaterals were of thinner caliber than the main axon and typically ended in clusters or basket-like terminations with several large (~1 μm) swellings characteristic of synaptic boutons. Some collaterals bore varicosities for short distances along their trajectory that resembled en passant boutons. Such clusters of varicosities tended to be absent along the length of the main axon and along most long stretches of collaterals as well. Axon collaterals were observed to terminate both within and beyond the dendritic fields of the parent neurons. Overall, the morphological similarities exhibited by these neurochemically diverse

The Journal of Comparative Neurology. DOI 10.1002/cne

MORPHOLOGY AND PHYSIOLOGY OF NIGRAL GABAERGIC NEURONS 963
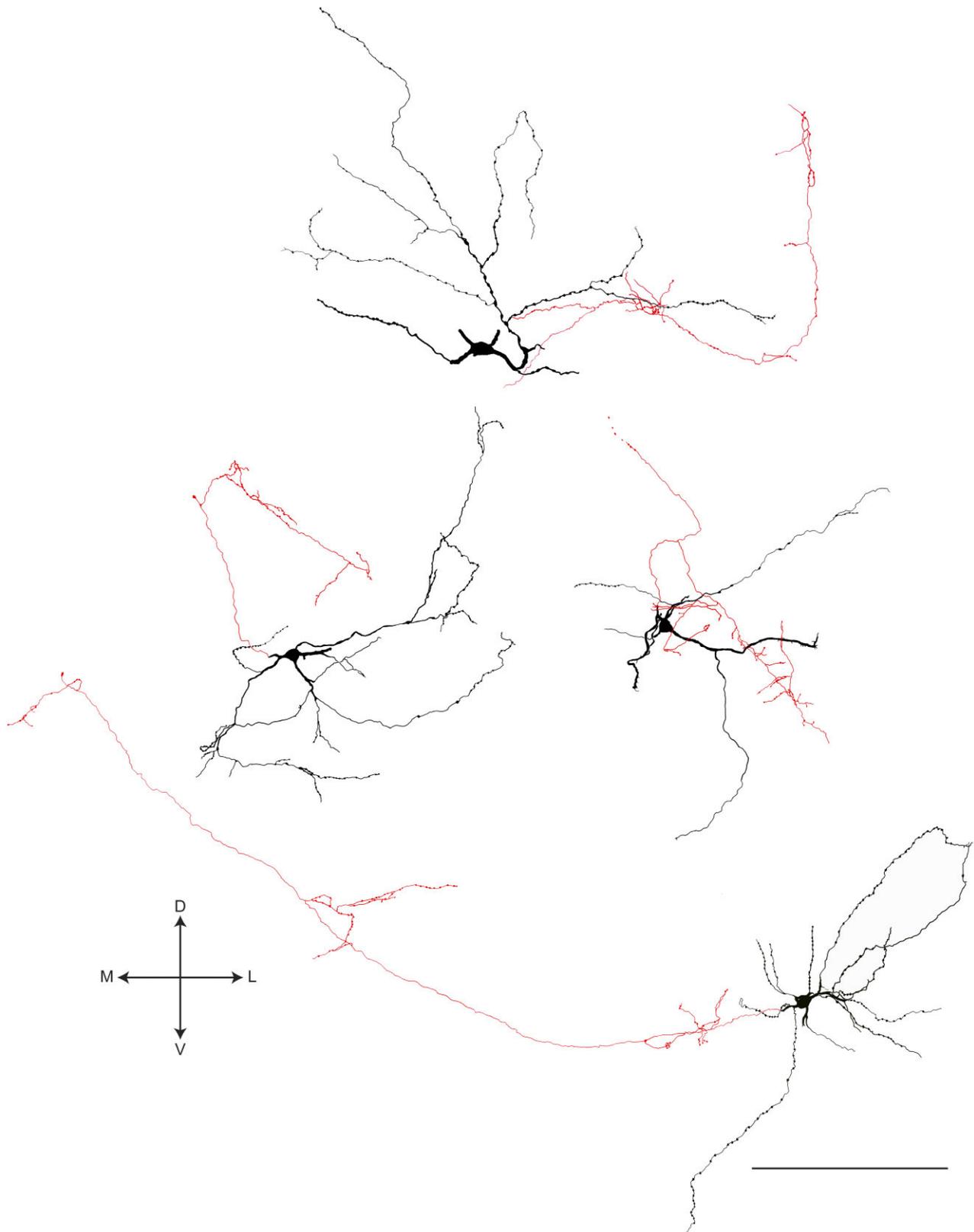
Fig. 2. Reconstructions of nigral GABAergic neurons exhibiting immunoreactivity for parvalbumin. A feature common to these neurons was the issuance of local axon collaterals, which is a property shared by all of the neurons. In these reconstructions and those to follow, the soma and dendrites are shown in black, and the axon in red. Scale bar = 200 μm.

The Journal of Comparative Neurology. DOI 10.1002/cne

964   C.R. LEE AND J.M. TEPPER
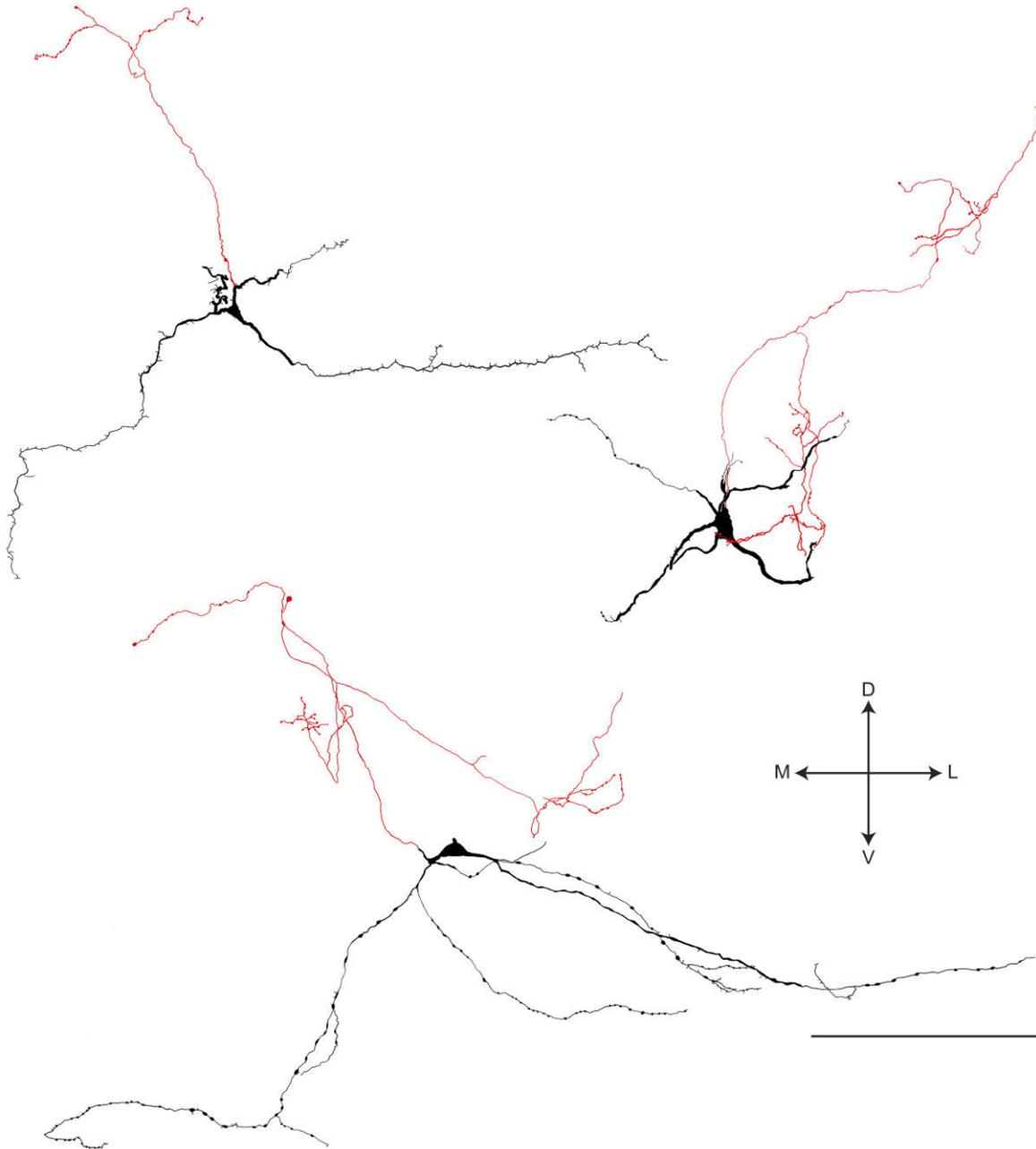
Fig. 3. Reconstructions of nigral GABAergic neurons exhibiting immunoreactivity for calretinin. Like the parvalbumin-immunoreactive neurons, calretinin-immunoreactive neurons also issued local axon collaterals. Scale bar = 200 μm.

neurons make it unlikely that these neurons can be distinguished based on morphological properties alone.

Physiological properties of immunocytochemically identified neurons

All of the neurons recorded (n = 55) fired regularly and spontaneously at rest (mean 17.94 ± 0.89 Hz, range 6.69–39.43 Hz). Unlike nigral dopaminergic neurons, which have a strong I_h and exhibit a pronounced sag in response to hyperpolarizing current pulses, GABAergic neurons exhibited a much less pronounced sag in response to hyperpolarizing current pulses (Nakanishi et al., 1987; Lacey et al., 1989; Yung et al., 1991; Richards et al., 1997; Gulacsi et al., 2003; Atherton and Bevan, 2005; Ibanez-Sandoval et al., 2006). However, sag amplitudes in nigral GABAergic neurons did show some variability and ranged from 1.11 to 15.40 mV (mean 6.23 ± 0.46 mV; Table 2, Fig. 4). Other properties included a short-duration spike (mean 0.33 ± 0.01 msec, range 0.20–0.55 msec), and a spike afterhyperpolariza-

The Journal of Comparative Neurology. DOI 10.1002/cne

MORPHOLOGY AND PHYSIOLOGY OF NIGRAL GABAERGIC NEURONS 965

TABLE 1. Summary of the Morphometric and Dendritic Characteristics of Immunocytochemically Identified Nigral GABAergic Neurons and Those That Did Not Exhibit Immunoreactivity for Either Parvalbumin or Calretinin[1]

Immunoreactivity	Area (μm²)	Perimeter (μm)	Shape factor	Primary dendrites	Sholl intersections	Longest extent (μm)	Dendritic tips
Parvalbumin (n = 22)	164.75 ± 7.65	53.57 ± 1.34	0.72 ± 0.02	4.3 ± 0.2 (n = 12)	64.4 ± 6.2 (n = 12)	313.3 ± 17.5 (n = 12)	15.3 ± 1.6 (n = 12)
Calretinin (n = 14)	186.42 ± 9.30	58.59 ± 1.23	0.68 ± 0.03	3.6 ± 0.5 (n = 8)	53.2 ± 6.6 (n = 8)	297.5 ± 38.8 (n = 8)	12.0 ± 1.6 (n = 8)
Neither (n = 10)	176.83 ± 14.48	56.22 ± 2.36	0.70 ± 0.02	—	—	—	—
Parvalbumin and calretinin (n = 1)	126.68	44.36	0.81	4	55	240	13

[1]No significant differences were found in the morphometric or dendritic characteristics exhibited by neurons with different calcium-binding proteins. The morphometric and dendritic characteristics of the single neuron that was observed to exhibit immunoreactivity for both calretinin and parvalbumin are also shown.

TABLE 2. Summary of the Electrophysiological Characteristics of Immunocytochemically Identified Nigral GABAergic Neurons and Those That Did Not Exhibit Immunoreactivity for Either Parvalbumin or Calretinin[1]

Immunoreactivity	Input resistance (MΩ)	AP threshold (mV)	AP amplitude (mV)	AP width (msec)	After hyperpolarization amplitude (mV)	After hyperpolarization latency (msec)	Firing rate (Hz)	Sag amplitude (mV)
Parvalbumin (n = 25)	313.64 ± 16.69	−51.58 ± 0.86	69.23 ± 1.21	0.32 ± 0.01	22.88 ± 0.92	1.17 ± 0.06	19.86 ± 1.51	6.12 ± 0.64
Calretinin (n = 16)	413.13 ± 31.31[2]	−49.55 ± 1.88	68.10 ± 1.74	0.36 ± 0.02	20.92 ± 1.51	1.40 ± 0.13	16.57 ± 1.52	7.16 ± 0.94
Neither (n = 13)	311.81 ± 33.27	−51.35 ± 1.66	73.55 ± 2.12	0.35 ± 0.02	22.62 ± 1.40	1.38 ± 0.15	15.85 ± 1.29	5.57 ± 0.89
Parvalbumin and calretinin (n = 1)	264.34	−51.09	73.42	0.24	22.72	0.82	19.23	2.44

[1]The electrophysiological characteristics of neurons with different calcium-binding proteins were largely similar, with the exception that the input resistance of calretinin-positive neurons was significantly higher than that observed in the other cell types. The electrophysiological characteristics of the single neuron that was observed to exhibit immunoreactivity for both calretinin and parvalbumin are also shown.
[2]$P = 0.01$.
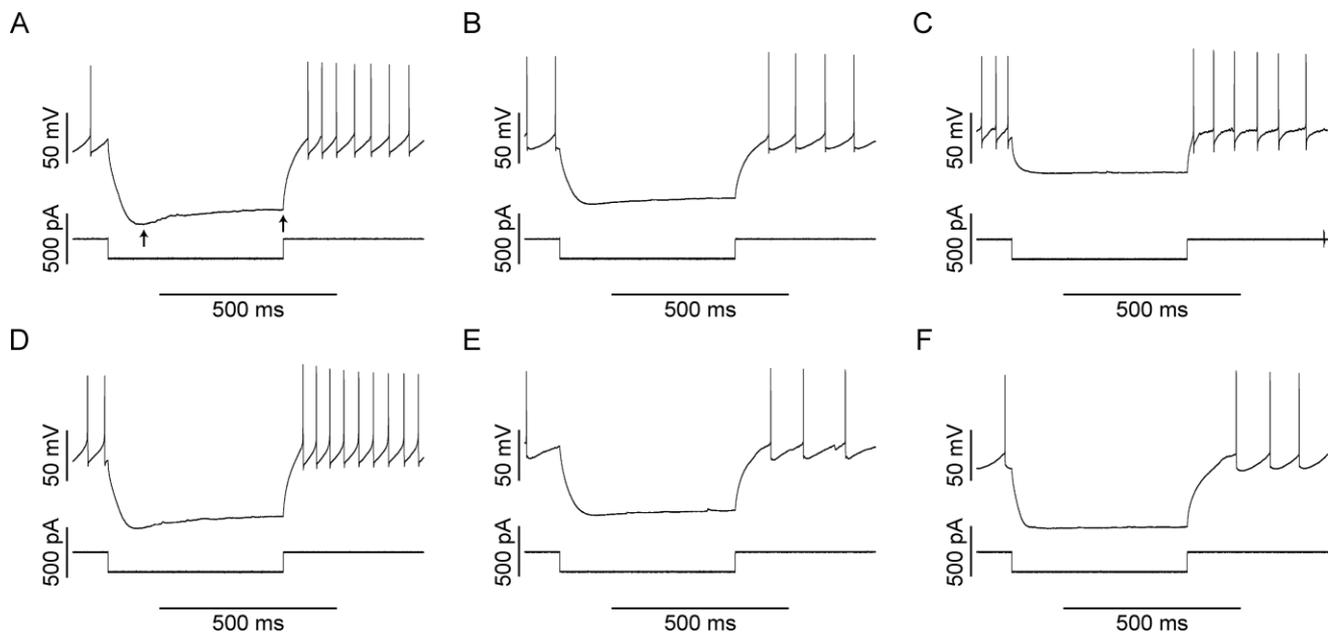
Fig. 4. Nigral GABAergic neurons exhibit variability in their responses to hyperpolarizing current pulses. Among the parvalbumin-immunoreactive neurons, some exhibited a pronounced sag (in **A**), whereas others exhibited intermediate levels (**B**), and some exhibited nearly none (**C**). Similar variations in sag amplitudes were observed in the calretinin-immunoreactive neurons; some exhibited a pronounced sag (in **D**), whereas others exhibited intermediate levels (**E**), and still others exhibited nearly none (**F**). Despite these variations, mean sag amplitudes were not significantly different among immunocytochemically identified neuron groups. The sag amplitude was calculated as the difference between the most hyperpolarized voltage achieved during the current pulse and the steady-state voltage measured 500 μsec prior to current offset as indicated by the arrows in A.

tion with a small amplitude (mean 22.25 ± 0.69 mV, range 12.25–35.62 mV) and a short time to trough (mean 1.28 ± 0.06 msec, range 0.76–3.09 msec), all properties previously attributed to nigral GABAergic projection neurons (Nakanishi et al., 1987; Lacey et al., 1989; Yung et al., 1991; Richards et al., 1997; Gulacsi et al., 2003; Atherton and Bevan, 2005). Other parameters measured included input resistance (mean 341.25 ± 15.29 MΩ, range 135.42–579.49 MΩ), action potential threshold (mean –50.93 ± 0.77 mV, range –64.99 to –32.58 mV), and action potential amplitude (mean 70.0 ± 0.93 mV, range 54.35–89.98 mV). The physiological properties of the immunocytochemically identified neurons are summarized in Table 2 and shown in Figures 5C, 6C, and 7C.

The Journal of Comparative Neurology. DOI 10.1002/cne

966   C.R. LEE AND J.M. TEPPER
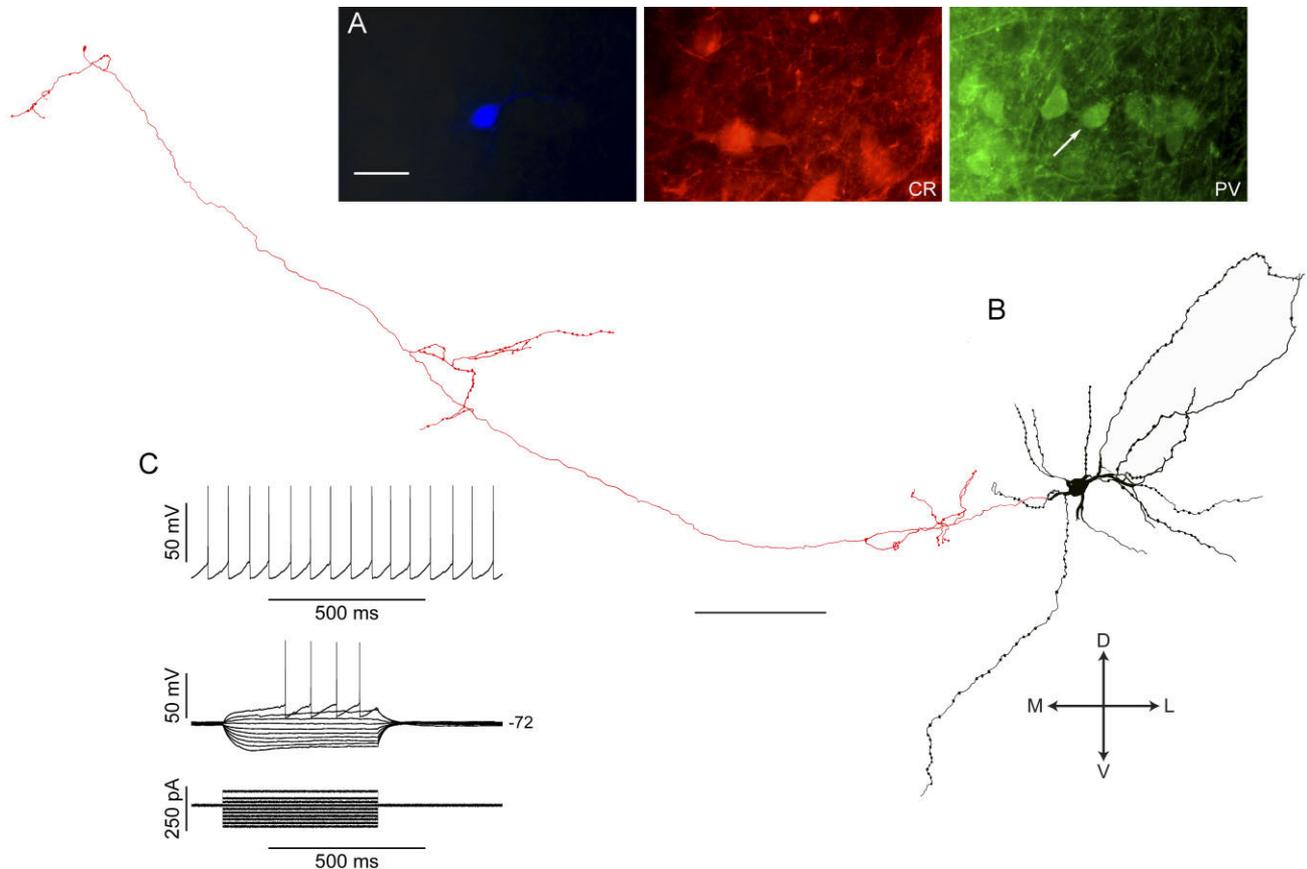
Fig. 5. Neurophysiology and morphology of a representative parvalbumin-immunoreactive neuron. **A:** This neuron was devoid of immunoreactivity for calretinin but exhibited immunoreactivity for parvalbumin (arrow). **B:** The issuance of several axon collaterals along the length of the axon is evident in the reconstruction of the neuron. **C:** The electrophysiological properties exhibited by this neuron were typical of those exhibited by all parvalbumin-positive neurons. In this and the following figures, the physiology shown includes 1 second of spontaneous activity (top) and the responses of the neuron to hyperpolarizing and depolarizing current pulses from a hyperpolarized holding potential sufficient to halt spiking (bottom). The physiological properties exhibited by this and the other neurons presented are characteristic of those observed in nigral GABAergic neurons, including regular spiking spontaneous activity at rest, a short-latency and short-duration spike afterhyperpolarization, a narrow spike width, and little time-dependent inward rectification in response to hyperpolarizing current pulses. Orientation refers to the reconstructed neuron. CR, calretinin; PV, parvalbumin. Scale bars = 25 μm in A; 100 μm in B.

The physiological properties of parvalbumin- or calretinin-immunoreactive neurons or neurons that were not immunoreactive for either were essentially identical, except that calretinin-immunoreactive neurons exhibited a higher average input resistance than that observed in either parvalbumin-positive neurons or those devoid of immunoreactivity [$F_{(2,51)}$ = 5.00, P = 0.01]. The single neuron immunoreactive for both parvalbumin and calretinin was not included in the statistical analyses, but its physiological properties fell within the ranges exhibited by neurons of the other types.

Combined immunocytochemistry, reconstruction, and electrophysiology of nigral GABAergic neurons

Figure 5A shows a typical example of a parvalbumin-immunoreactive neuron. As was common for all neurons in our sample, this neuron issued several axon collaterals from its main axon. The collaterals made small arborizations expressing both terminal and en passant boutons in three discrete regions separated by several hundred micrometers (Fig. 5B). As was also common for the neurons recorded, this cell exhibited regular spiking spontaneous activity at rest (Fig. 5C).

Figure 6A shows a typical example of a calretinin-immunoreactive neuron. It, too, issued axon collaterals that expressed small clusters of prominent boutons in discrete areas of substantia nigra separated by hundreds of micrometers (Fig. 6B). As with the parvalbumin-immunoreactive neuron, this neuron exhibited regular spiking spontaneous activity (Fig. 6C).

The only neuron exhibiting both parvalbumin and calretinin immunoreactivity is shown in Figure 7A. Its morphological and electrophysiological characteristics were indistinguishable from those of neurons singly immunoreactive for either parvalbumin or calretinin (Fig. 7B and C, respectively).
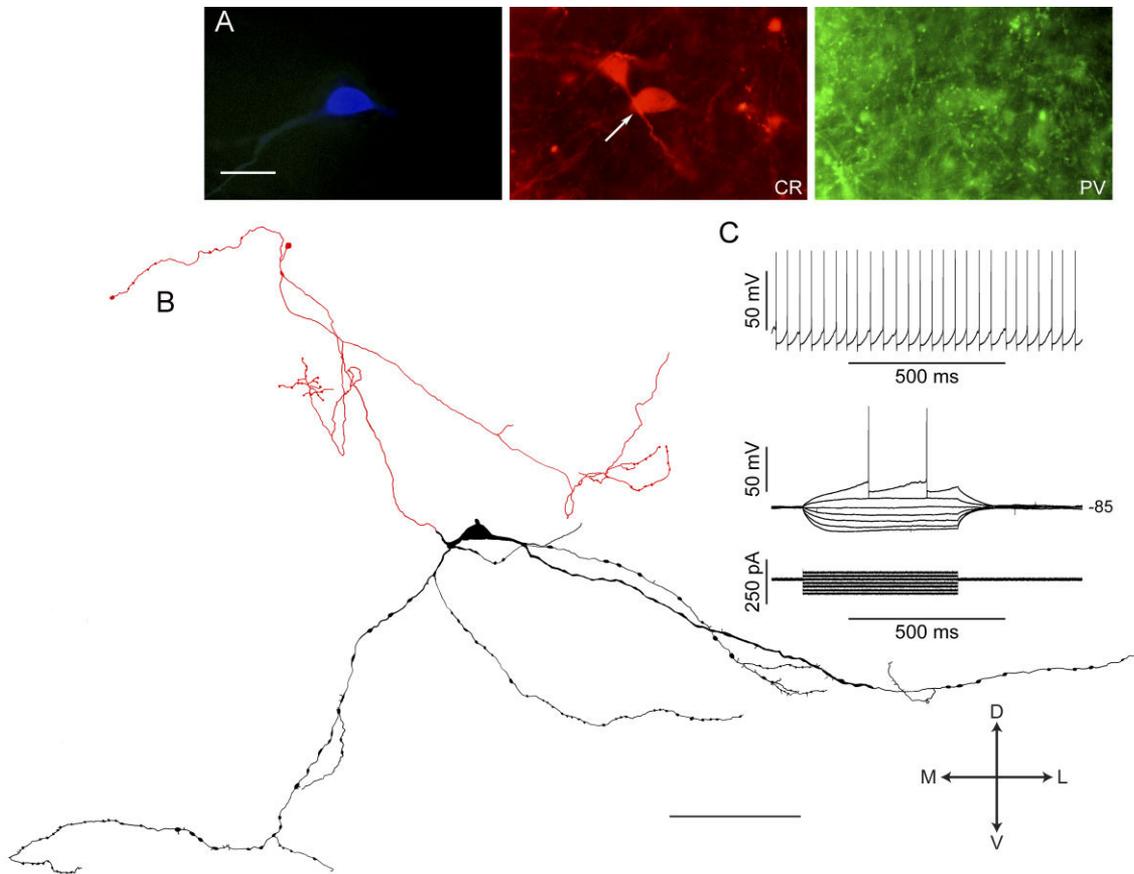
Fig. 6. Neurophysiology and morphology of a representative calretinin-immunoreactive neuron. **A:** This neuron exhibited immunoreactivity for calretinin (arrow) but was devoid of immunoreactivity for parvalbumin. **B:** Like the parvalbumin-immunoreactive neuron shown in the previous figure, this neuron issued prominent axon collaterals. **C:** The electrophysiological properties of this neuron were typical of those exhibited by calretinin-positive neurons and did not differ from those exhibited by the parvalbumin-positive neurons. CR, calretinin; PV, parvalbumin. Scale bars = 25 μm in A; 100 μm in B.

Projection sites of immunocytochemically identified neurons

The aforementioned results reveal that nigral GABAergic neurons containing either parvalbumin or calretinin are physiologically and morphologically similar or identical. To determine whether these neurons differ in their distal targets, the retrograde tracer fluorogold was injected into either the superior colliculus (n = 7) or the ventral anterior thalamus (n = 5), followed by immunocytochemistry for parvalbumin (superior colliculus n = 3; thalamus n = 3) or calretinin (superior colliculus n = 4; thalamus n = 2) to identify their constituent calcium-binding proteins.

Dense retrograde labeling from the superior colliculus was generally confined to ventrolateral peripeduncular regions of the rostral substantia nigra, but labeled perikarya could be found in other parts of the nucleus as well. Fluorogold injections into the ventral anterior thalamus led to labeling of perikarya throughout the SNr. There was no correlation between calcium-binding protein expression and terminal fields for the substantia nigra projection neurons. Both parvalbumin-immunoreactive neurons and calretinin-immunoreactive neurons projected nonpreferentially to tectum or to thalamus, as shown in Figure 8.

DISCUSSION

The results presented here suggest that the GABAergic neurons of the substantia nigra are largely homogeneous in terms of their morphological and physiological properties, despite exhibiting differences in immunoreactivity for the calcium-binding proteins parvalbumin and calretinin. Furthermore, it is clear that intranigral communication is ubiquitously carried out by both parvalbumin- and calretinin-containing neurons as well as a small population of neurons containing both parvalbumin and calretinin. The morphology and distribution of the axon collaterals of nigral GABAergic neurons have been described in detail previously in the elegant in vivo studies of Mailly and colleagues (2003), but the present experiments are the first to reveal the calcium-binding protein expression of the neurons issuing those collaterals. The presence of axon collaterals in substantia nigra is a feature unique to the GABAergic neurons, insofar as axon collaterals are never observed to emerge from axons of dopaminergic neurons (Juraska et al., 1977; Tepper et al., 1987).

In most brain regions, interneurons are electrophysiologically, morphologically, and/or neurochemically distinct from the principal neurons. In the present study,

The Journal of Comparative Neurology. DOI 10.1002/cne

968   C.R. LEE AND J.M. TEPPER
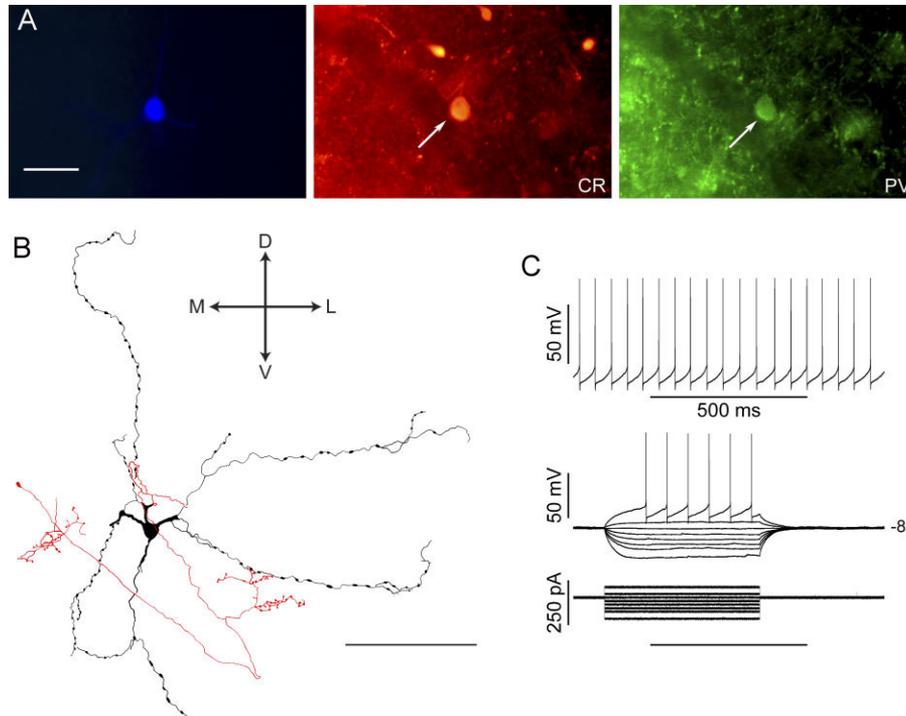
Fig. 7. Neurophysiology and morphology of the only neuron in our sample that exhibited immunoreactivity for both parvalbumin and calretinin. **A:** This neuron was immunoreactive for both parvalbumin and calretinin (arrows). **B:** As with the other neurons, several axon collaterals can be seen to issue from the main axon. **C:** Although neurochemically unique, this neuron did not exhibit electrophysiological characteristics that differed from those observed in singly immunoreactive neurons. CR, calretinin; PV, parvalbumin. Scale bars = 25 μm in A; 100 μm in B.

however, all the nigral neurons were morphologically and electrophysiologically similar. In addition, all issued local axon collaterals that exhibited a small number of discrete areas giving rise to clusters of varicosities, separated by long nonvaricose stretches (see Figs. 2, 3). This pattern of axonal arborization has been shown in in vivo labeling studies to be exhibited by nigrothalamic and nigrotectal projection neurons (Deniau et al., 1982; Grofova et al., 1982; Tepper et al., 2002; Mailly et al., 2003). Thus, although we cannot rule out the possibility that there are specialized GABAergic interneurons projecting locally within the substantia nigra, they are either both morphologically and electrophysiologically indistinguishable from the projection neurons or exist in such small numbers that we were unable to detect them despite a concerted effort.

Significance of intranigral communication between GABAergic and dopaminergic neurons

The inhibition of substantia nigra dopaminergic neurons by nigral GABAergic neurons (MacNeil et al., 1978; Walters and Lakoski, 1978; Grace and Bunney, 1979, 1985a,b; Waszczak et al., 1980; Hajos and Greenfield, 1994; Hausser and Yung, 1994; Paladini et al., 1999; Saitoh et al., 2004) is a key element of nigral microcircuitry and may represent the most efficacious source of GABAergic modulation of those neurons (Tepper et al., 1995; Celada et al., 1999; Lee et al., 2004). Numerous studies have established that GABAergic input to the nucleus often results in inhibition of nigral GABAergic neurons and disinhibition of nigral dopaminergic neurons (Waszczak et al., 1980; Collingridge and Davies, 1981; Grace and Bunney, 1985a; Celada et al., 1999; Lee et al., 2004), supporting the notion that GABAergic inputs to the substantia nigra can function disynaptically, through the GABAergic neurons of the SNr. As shown here, both parvalbumin- and calretinin-containing neurons issue axon collaterals possessing varicosities that are likely to be synaptic boutons that participate in intranigral communication.

Significance of intranigral communication among GABAergic and other GABAergic neurons

Although less well studied than the interaction between nigral GABAergic and dopaminergic neurons, local inhibitory interactions between GABAergic neurons in the substantia nigra have also been documented (Karabelas and Purpura, 1980; Deniau et al., 1982; Nitsch and Riesenberg, 1988; Hajos and Greenfield, 1994; Rick and Lacey, 1994; Shen and Johnson, 1997; Gulacsi et al., 2003). The SNr has been shown to be divided into functional subregions based on afferent GABAergic input from similarly segregated functional subregions of the striatum (Deniau et al., 1996; Mailly et al., 2001). The often relatively long distances observed between the cell body and the axon collaterals of nigral GABAergic neurons suggest that there may be communication between different subregions of the SNr through axon collaterals. Given such a situation, it is possible that inhibition of a GABAergic
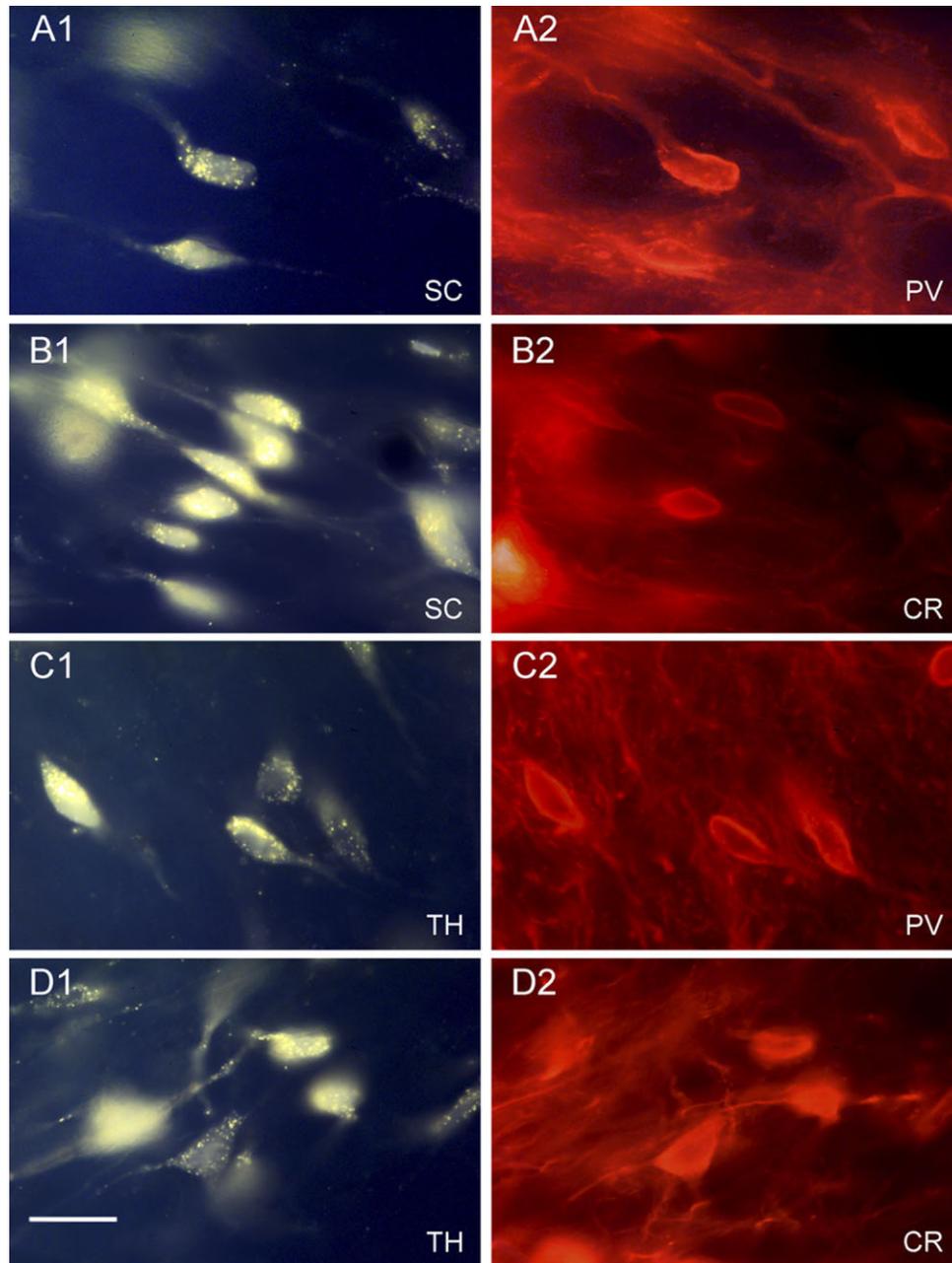
Fig. 8. Projection targets of parvalbumin- and calretinin-immunoreactive neurons. Retrograde labeling of nigral projection neurons is shown from the superior colliculus (**A1,B1**) and thalamus (**C1,D1**). Neurons projecting to the superior colliculus were observed to exhibit immunoreactivity for parvalbumin (**A2**) or calretinin (**B2**). Similarly, neurons projecting to the thalamus also exhibited immunoreactivity for parvalbumin (**C2**) or calretinin (**D2**). CR, calretinin; PV, parvalbumin; SC, superior colliculus; TH, thalamus. Scale bar = 25 μm.

projection neuron by the striatum or globus pallidus, in addition to disinhibiting target neurons, could lead to disinhibition of other GABAergic neurons in the SNr through axon collaterals, leading to increased inhibition of target surround neurons in the thalamus or superior colliculus as well as increased inhibition of distant as well as neighboring neurons in the SNr (Chevalier and Deniau, 1990).

Significance of calcium-binding protein heterogeneity between nigral GABAergic neurons

The present experiments have demonstrated a stark contrast between the heterogeneity of calcium-binding proteins that is present among nigral GABAergic neurons that exhibit immunoreactivity for parvalbumin or

calretinin, as well as a small population which exhibits both, and the homogeneity of physiological and morphological characteristics and projection sites observed. It is clear that, unlike the case in other basal ganglia nuclei such as the neostriatum (Kawaguchi, 1993; Kawaguchi et al., 1995; Tepper and Bolam, 2004) and other regions such as the hippocampus (Freund and Buzsaki, 1996; Somogyi and Klausberger, 2005) and neocortex (DeFelipe, 1997), expression of parvalbumin and calretinin in the substantia nigra is not confined to intrinsic interneurons. In the substantia nigra, these calcium-binding proteins are instead localized within projection neurons that are morphologically and physiologically similar and likely mediate local interactions through their axon collaterals in addition to inhibiting neurons in target nuclei.

The significance of the neurochemical heterogeneity observed here is unclear. One possibility is that nigral GABAergic neurons containing parvalbumin or calretinin are derived at different ontogenetic time points. A relationship between calcium-binding protein expression and ontogenesis has been shown to exist in the substantia nigra, with the expression patterns of calretinin and parvalbumin differing over time (Alfahel-Kakunda and Silverman, 1997). It seems plausible that calretinin and parvalbumin might be localized in nigral GABAergic neurons that originate from different developmental intervals but are otherwise identical. Alternatively, the calcium-binding proteins may have presynaptic actions and differentially affect transmitter release in terminal regions (Caillard et al., 2000; Edmonds et al., 2000).

CONCLUSIONS

The GABAergic neurons of the SNr are heterogeneous in terms of their calcium-binding proteins, and most express immunoreactivity for parvalbumin or calretinin, and a small population expresses both proteins. Despite this neurochemical heterogeneity, the neurons are physiologically, morphologically, and hodologically homogeneous. Nigral GABAergic neurons expressing parvalbumin, calretinin, or both proteins possess local axon collaterals, which likely underlie the intranigral communication that has been described between GABAergic and dopaminergic neurons and among GABAergic neurons themselves.

ACKNOWLEDGMENTS

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972   **C.R. LEE AND J.M. TEPPER**

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