



IMMUNOHISTOCHEMICAL LOCALIZATION OF *N*-METHYL-D-ASPARTATE AND α -AMINO-3-HYDROXY-5-METHYL-4-ISOXAZOLEPROPIONATE RECEPTOR SUBUNITS IN THE SUBSTANTIA NIGRA PARS COMPACTA OF THE RAT

D. S. ALBERS,* S. W. WEISS,* M. J. IADAROLA† and D. G. STANDAERT*‡

*Neurology Service, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, U.S.A.

†Pain and Neurosensory Mechanisms Branch, National Institute of Dental Research, NIH, Bethesda, MD 20892, U.S.A.

Abstract—Ionotropic glutamate receptors in the substantia nigra pars compacta regulate the activity of dopamine neurons. We have used dual-label immunofluorescence and confocal laser microscopy to study the localization of subunits of two types of ionotropic receptors within the substantia nigra pars compacta of the rat. Immunostaining for *N*-methyl-D-aspartate receptor 1 and glutamate receptor 2/3 was prominent in the soma and proximal dendrites of all tyrosine hydroxylase-immunopositive cells, while only low amounts of *N*-methyl-D-aspartate receptor 2A and *N*-methyl-D-aspartate receptor 2B were present. Selective antibodies were used to determine the isoforms of *N*-methyl-D-aspartate receptor 1 present. Immunostaining for the N1, C1 and C2 variably spliced segments of *N*-methyl-D-aspartate receptor 1 were scarce in the substantia nigra pars compacta, while immunoreactivity for the alternative C2' terminus of *N*-methyl-D-aspartate receptor 1 was quite abundant. Staining for glutamate receptor 1 was heterogeneous; about half of the tyrosine hydroxylase immunopositive cells stained intensely, while the other half were immunonegative. The glutamate receptor 1-stained cells were concentrated in the ventral tier of the substantia nigra pars compacta. Glutamate receptor 4 was not found in tyrosine hydroxylase-immunopositive cells within the substantia nigra pars compacta.

Together, these data demonstrate that dopaminergic neurons in the substantia nigra pars compacta express primarily glutamate receptor 1, glutamate receptor 2/3 and *N*-methyl-D-aspartate receptor 1 isoforms containing the alternative C2' terminus. © 1998 IBRO. Published by Elsevier Science Ltd.

Key words: dopamine, confocal microscopy, Parkinson's disease, basal ganglia, glutamate.

Neurons of the substantia nigra pars compacta (SNpc) provide dopaminergic innervation to the striatum (caudate and putamen). Progressive degeneration of this nigrostriatal pathway causes the common human neurological disorder, Parkinson's disease (PD). The excitatory transmitter glutamate is thought to modulate the activity of these cells and may have a role in their selective death in PD.^{4,5} Of the several types of glutamate receptors, those most closely linked to the regulation of the nigrostriatal pathway are two families of ionotropic receptor: *N*-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). Electrophysiological and pharmacological evidence suggests that, in the rat, receptors of both types are

located on the soma and dendrites of dopaminergic neurons and regulate their electrical activity.^{9,10} The rodent SNpc contains abundant NMDA- and AMPA-type glutamate binding sites,¹ but the cellular localization and structural nature of the receptors responsible are not known.

Recent advances in molecular biology have greatly improved our understanding of the structure, properties and expression of NMDA and AMPA receptors. NMDA receptor subunits are encoded by two families of genes, designated *N*-methyl-D-aspartate receptor 1 (NMDAR1) and NMDAR2.^{19,34} The NMDAR1 class comprises eight splice variants of a single gene, while the NMDAR2 class consists of four different gene products (NMDAR2A–D).^{21,31,51} Isoforms of NMDAR1 are produced by alternative splicing of three cassettes encoding a short, 21-amino-acid segment in the amino terminus (termed N1), as well as two longer, adjacent segments in the carboxy terminus (termed C1 and C2).^{11,17,33,44,51} Splicing out the region encoding the C2 segment removes the first stop codon and results in an extended open reading frame that encodes an

‡To whom correspondence should be addressed.

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate; DA, dopamine; FITC, fluorescein isothiocyanate; GluR, glutamate receptor subunit; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor subunit; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

Table 1. Antibodies used for immunohistochemical localization of *N*-methyl-D-aspartate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor subunit proteins

Specificity	Identifier	Antigen	Amino acids	Dilution	Reference
NMDAR1	54.1	Fusion protein	660–811	1:250	41
NMDAR1, N1	22282	Peptide	1–21 of exon 5	0.5 μ g/ml	40
NMDAR1, C1	17182	Peptide	864–900	0.5 μ g/ml	40
NMDAR1, C2	C2	Peptide	923–938	1:500	3
NMDAR1, C2'	C2'	Peptide	1–17 of C2' segment	1:800	20
NMDAR2A	2A	Fusion protein	1253–1391	1:500	14
NMDAR2B	2B	Fusion protein	984–1104	1:1000	14
GluR1	Ab7	Peptide	877–889	1:200	50
GluR2/3	Ab25	Peptide	850–862	1:200	50
GluR4	Ab22	Peptide	868–881	1:200	50

Listed are the specificity, identifying name or number, type of antigen, and working dilution for the NMDA and AMPA receptor subunit protein antibodies used in this study. All antibodies were raised in rabbits except NMDAR1, which was raised in mouse. The references listed describe these antibodies in more detail.

alternative carboxy terminal sequence of 22 amino acids (termed C2').⁵¹ AMPA receptor complexes are also heteromeric assemblies of subunits composed of combinations of four proteins (glutamate receptor subunits 1–4, GluR1–GluR4) encoded by four distinct genes.⁵⁰ *In vitro*, the pharmacological and electrophysiological properties of heteromeric NMDA and AMPA receptors vary depending on the subunits present.^{7,11,17,19,21,31,32,34,50}

To determine which glutamate receptor subunits were present in dopaminergic neurons of the SNpc in the rat, we used dual-label immunofluorescence and confocal laser microscopy to study the localization of NMDAR1, its variably spliced segments (N1, C1, C2 and C2'), NMDAR2A, NMDAR2B and GluR1–GluR4 receptor proteins in these neurons, identified by the presence of tyrosine hydroxylase (TH).

EXPERIMENTAL PROCEDURES

Antibodies and antisera

All antisera used in this study have been characterized previously (see Table 1). Two affinity purified antibodies were obtained from Dr Morgan Sheng, of the Neurobiology Department and Howard Hughes Medical Institute at Massachusetts General Hospital. These antibodies were produced using peptide immunogens in rabbits and are designated 22282 (for the N1 segment of NMDAR1) and 17182 (C1 segment of NMDAR1). These antibodies have been characterized extensively by immunoblotting techniques with extracts of rat brain.⁴⁰ The rabbit antiserum to the C2 segment of NMDAR1 was obtained from Dr Ted Dawson of Johns Hopkins University. This antiserum has been characterized by immunoblot analysis of rat brain extracts and NMDAR1 protein expressed in transfected cells, and used in several previous immunohistochemical studies of the rat visual cortex.^{2,3} The mouse monoclonal

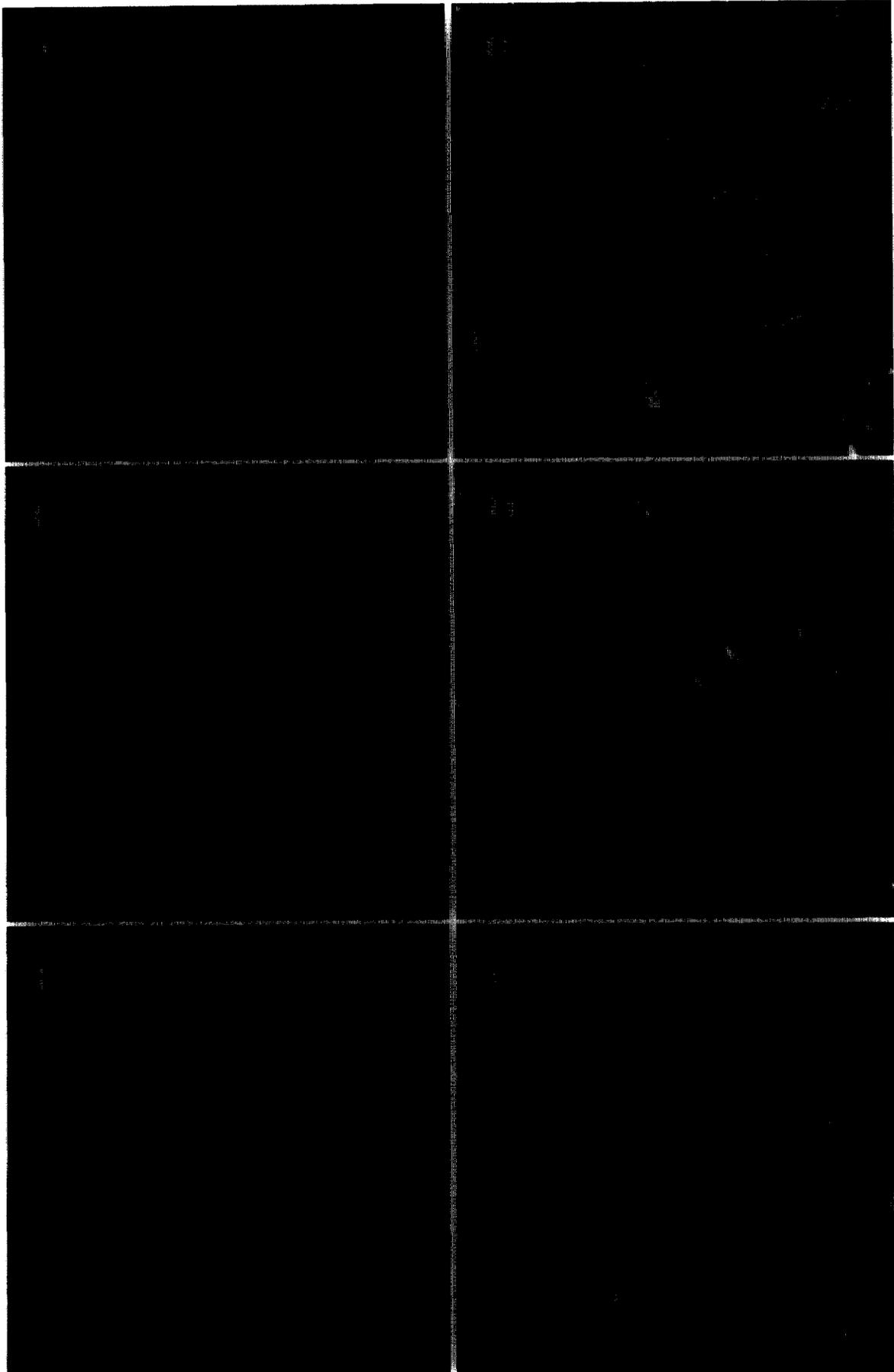
antibody to NMDAR1 (54.1) was purchased from Pharmingen (San Diego, CA) and has been characterized previously.⁴¹ The rabbit polyclonal antibodies to NMDAR2A and NMDAR2B were purchased from Chemicon (Temecula, CA).¹⁴ The rabbit polyclonal antibodies to GluR1, GluR2/3 and GluR4 were provided by Dr Robert Wenthold and have been described previously.^{38,50} A mouse monoclonal antibody to rat⁴ TH (TH-2 clone) was purchased from Sigma Chemical Co. (St Louis, MO), while a rabbit polyclonal antibody to TH was obtained from Chemicon (AB152).

Immunohistochemistry

Dual-label immunohistochemistry was conducted as described previously.^{42,45,49} Experiments were conducted in male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA; 250–300 g) in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and were approved by the MGH Subcommittee on Research Animal Care. Rats were deeply anesthetized with pentobarbital and perfused with normal saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.9% NaCl (PBS), at room temperature. The brains were removed, postfixed for 1 h, cryoprotected overnight in 30% sucrose at 4°C and frozen in isopentane cooled with dry ice. Sections (50 μ m) were cut using a freezing microtome and either processed immediately for immunohistochemistry or stored in 50% glycerol in 100 mM Tris (pH 7.5) at –20°C.

For immunohistochemistry, sections were washed in PBS, incubated in 3% normal goat serum with 0.3% Triton X-100 in PBS for 1 h, then incubated for 24–48 h at 4°C (NMDAR2A, NMDAR2B; GluR1–GluR4; N1, C1, C2, C2'; monoclonal TH) or room temperature (monoclonal NMDAR1; polyclonal TH), in the same solution containing primary antibodies to an NMDA receptor or AMPA receptor subunit in combination with an antibody to TH. Sections were then washed in PBS and incubated sequentially in two fluorescent secondary antibodies [one Cy3 conjugated and the other either fluorescein isothiocyanate (FITC) or Cy2 conjugated; Jackson Laboratories, West Grove, PA]. The sections were mounted on gelatin-coated slides, dried,

Fig. 1. Localization of NMDA receptor subunit immunoreactivity in TH-positive neurons of the SNpc. Panels on the left demonstrate immunohistochemical staining for NMDAR1 (A), NMDAR2A (C) and NMDAR2B (E). Panels on the right (B, D, F) illustrate staining of the same sections for TH. Staining for NMDAR1 is present in both TH-positive and TH-negative cells. The antibody to NMDAR2A or NMDAR2B produced low levels of staining in TH-positive cells. The staining pattern of NMDAR2A was limited to the cytoplasmic areas, while NMDAR2B immunostaining was more prominent in the neuropil. The arrows point to TH-positive cells stained lightly for NMDAR2A (C, D) and NMDAR2B (E, F). Scale bar = 50 μ m.



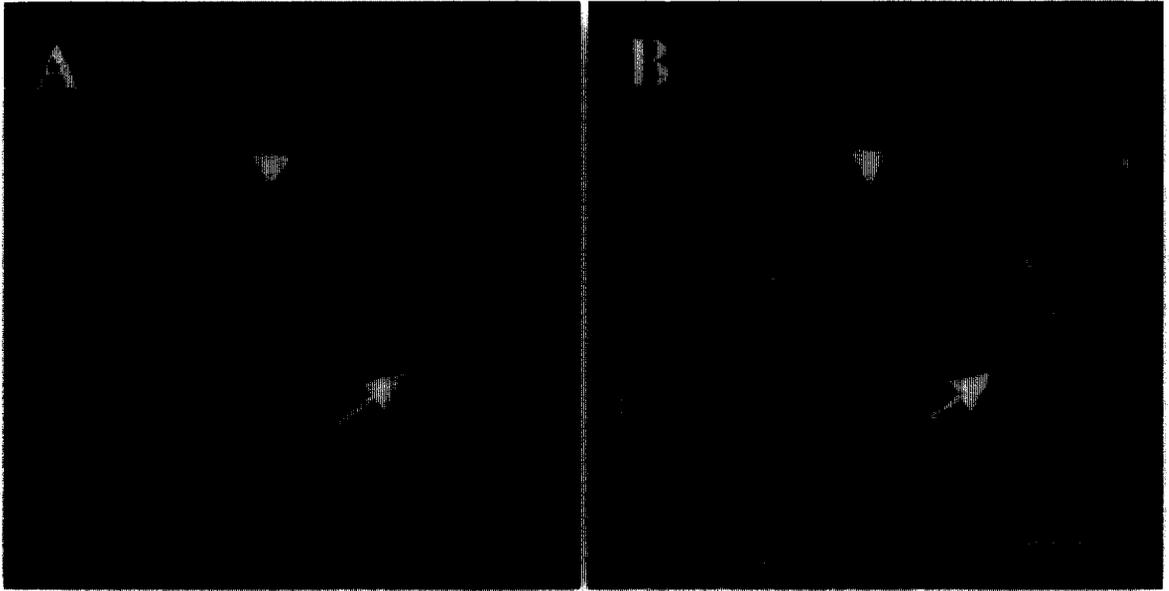


Fig. 2. High magnification of NMDAR1 immunoreactivity in TH-positive neurons of the SNpc. The panel on the left demonstrates immunostaining for NMDAR1 (A). The panel on the right illustrates staining of the same section for TH (B). NMDAR1 immunostaining is present in both TH-positive (arrows) and TH-negative neurons (arrowheads). Scale bar = 10 μ m.

and coverslipped using glycerol containing 100 mM Tris (pH 8.0) and 2% *p*-phenylenediamine (Sigma) to retard fading. Preparations were examined using a BioRad Laser Confocal system (MRC 1000) equipped with a Leica DMBR microscope and an argon/krypton laser. High-magnification images were obtained using an oil-immersion lens with a numerical aperture of 1.4. Images were obtained by illuminating the section with a single laser line and collecting the image using an appropriate emission filter: for Cy3, excitation at 568 nm and a 605-nm long-pass filter; for FITC or Cy2, excitation at 488 nm and a 522-nm band-pass filter. For each wavelength, four sequential images 1024 \times 1024 pixels in size with an eight-bit pixel depth were obtained and averaged, using a Kalman filtering method to reduce noise. Dual-label images were performed by collecting the separate images sequentially, and reconstructing the images in color using Adobe Photoshop software on a Macintosh computer.

Each experiment included control tissue, processed with omission of primary antibodies but with both Cy3 and FITC or Cy2 secondary antibodies. These sections were examined both by fluorescence microscopy and laser confocal microscopy, and exhibited no detectable signal. Pre-absorption controls were conducted for each of the antibodies for which such experiments have not been reported previously. The immunostaining patterns of the N1, C1 and C2' antibodies were eliminated after preincubation of these antisera with their respective peptide immunogens at a concentration of 1–10 μ M (data not shown).

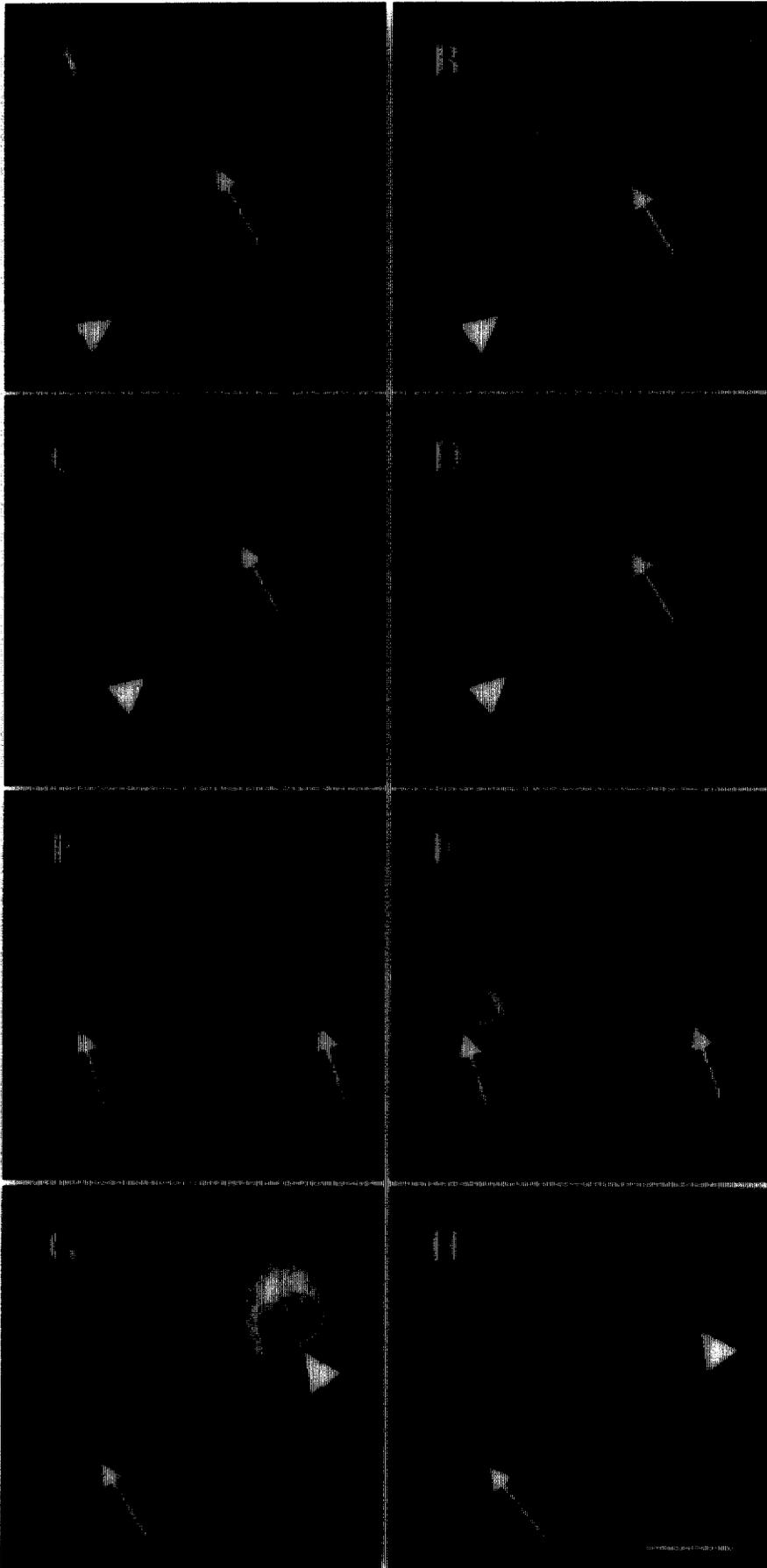
RESULTS

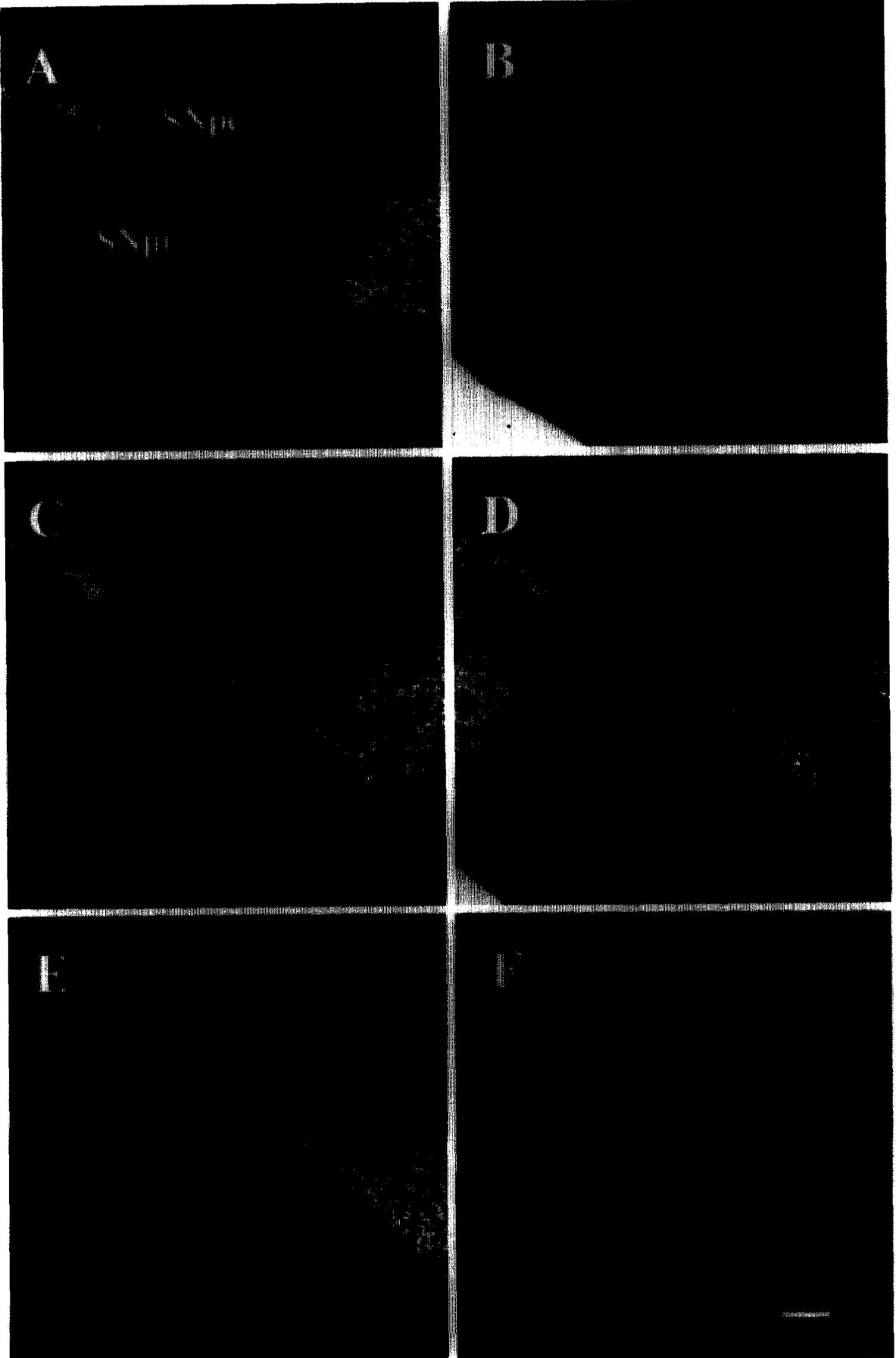
N-Methyl-D-aspartate receptor subunits in the substantia nigra pars compacta

The polyclonal antibody to TH produced intense labeling of the soma and dendrites of large neurons in the SNpc (Fig. 1). Dual-label experiments with the monoclonal NMDAR1 pan antibody revealed all of the TH-positive neurons to exhibit intense NMDAR1 immunoreactivity (Fig. 1A). The NMDAR1 immunostaining had a punctate appearance and was mostly abundant in the cytoplasm of neurons. Distinct clusters of NMDAR1 immunostaining could be detected throughout the cytoplasm of TH-positive neurons (Fig. 2). NMDAR1 immunostaining was also present in the neuropil. Some of the distinct, intensely labeled puncta appeared to be present within the large, TH-immunoreactive processes of SNpc neurons, while other puncta were not associated with TH-labeled structures.

Staining of the SNpc with the antibodies targeted to the alternatively spliced regions of NMDAR1 revealed that the TH-positive neurons exhibited a characteristic pattern of immunoreactivity.

Fig. 3. Presence of alternatively spliced segments of NMDAR1 in TH-positive neurons of the SNpc. The left panel illustrates the immunoreactivity of N1 (A), C1 (C), C2 (E) and C2' (G) splice segments of NMDAR1. The right panel (B, D, F, H) illustrates the staining for TH in the same sections. N1 immunoreactivity was very low in TH-positive cells (arrow; A, B). In contrast, adjacent TH-negative neurons were immunoreactive for N1 (arrowhead). Both antibodies to C1 and C2 produced little labeling of the TH-positive neurons (arrows; C, D and E, F, respectively). However, TH-negative cells contained immunoreactive puncta of C1 (arrowhead), whereas C2 intensely labeled fibers coursing through the region. Antiserum to C2' produced intense labeling of TH-positive cells (arrow) and TH-negative cells (arrowhead; G, H). Scale bar = 10 μ m.





TH-positive neurons, identified with the monoclonal antibody, exhibited only very rare N1-immunoreactive puncta (Fig. 3A). In contrast, many adjacent TH-negative neurons contained abundant N1 immunoreactivity. In these cells, there was intense staining of small immunoreactive puncta in the cytoplasm, along the plasma membrane and within proximal processes. There was also punctate staining of the neuropil. A similar pattern of staining was observed using the antibody to the C1 segment of NMDAR1; only a few immunoreactive puncta were observed in the cytoplasm and proximal processes of TH-positive neurons, while some of the adjacent TH-negative cells contained fairly numerous collections of these puncta (Fig. 3C). The antiserum to the C2 segment of NMDAR1 also produced very little labeling of the TH-positive neurons of the SNpc (Fig. 3E). However, it did produce intense labeling of a small number of beaded fibers coursing through the region. Similar beaded fibers were seen in other regions of the brain, particularly in the bed nucleus of the stria terminalis, as described recently using a different C2 segment antibody.¹⁶ The antiserum to the C2' segment of the NMDAR1 was the only splice-segment-specific antibody that produced intense labeling of TH-positive neurons (Fig. 3G). The staining pattern of this alternatively spliced region in TH-positive neurons was similar to that observed with the NMDAR1 pan antibody. There was intense C2' staining present throughout the soma and proximal dendrites of TH-positive neurons, and labeled puncta were observed within and along TH-positive processes throughout the SNpc.

Immunoreactivity for both NMDAR2A and NMDAR2B proteins was low in the SNpc; within TH-positive neurons, NMDAR2A and NMDAR2B immunostaining was weak (Fig. 1C, E), and was detected within the cytoplasm (NMDAR2A and NMDAR2B) and the neuropil (NMDAR2B). In the same preparations, neurons in the hippocampus and cerebral cortex were strongly immunoreactive for these receptor subunits (not illustrated).

α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor subunits in the substantia nigra pars compacta

The most striking feature of the GluR1 immunostaining within the SNpc was the heterogeneity in staining intensity of TH-positive neurons. Most of the GluR1 immunoreactivity was associated with TH-positive cells in the ventral tier of the SNpc (Fig. 4A, B). At higher magnification, intense GluR1 staining of the cytoplasm and dendritic processes of these

cells was evident (Fig. 5A, B). In contrast, many of the TH-positive cells in the more dorsal part of the SNpc contained little or no immunostaining for GluR1 (Fig. 4A, B). GluR2/3 immunostaining in the SNpc was also intense, but more homogeneously distributed (Fig. 4D). All of the TH-positive SNpc neurons examined exhibited somatic and dendritic staining for GluR2/3 (Fig. 5C, D). There was no observable GluR4 immunoreactivity associated with TH-positive cells in the SNpc (Fig. 4F). GluR4-immunoreactive cells were present in the substantia nigra pars reticulata, particularly the lateral part, but these were not stained for TH.

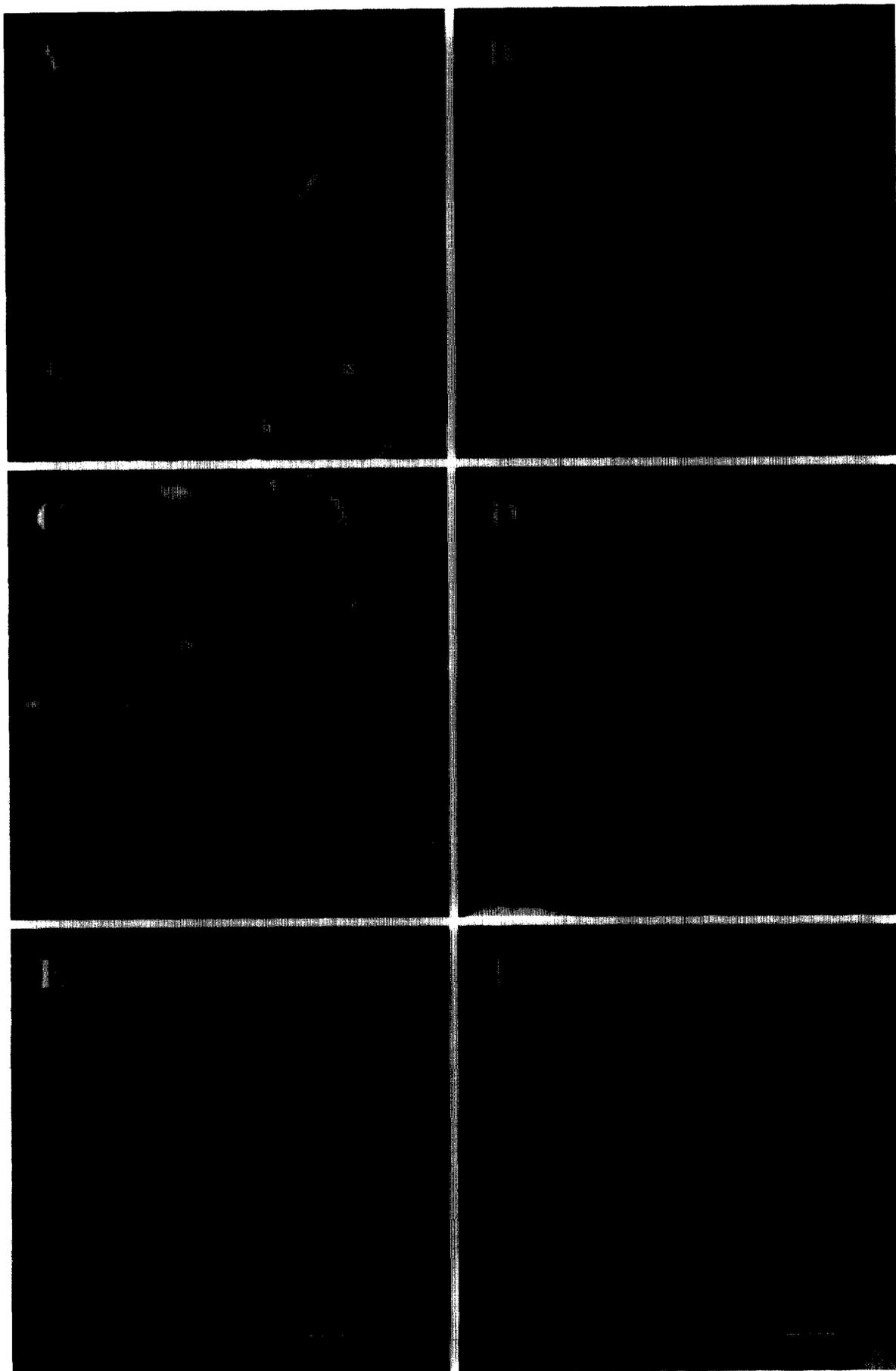
DISCUSSION

Using a well-characterized panel of antibodies to the NMDA and AMPA receptor subunits, we have found immunoreactivity for a subset of these proteins in the soma and proximal processes of TH-positive neurons in the SNpc. These experiments extend previous immunohistochemical observations and demonstrate directly the existence of NMDA and AMPA receptors on TH-positive cells in the rat SNpc, which has been inferred from electrophysiological and *in situ* hybridization experiments.

Localization of N-methyl-D-aspartate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate subunits in the substantia nigra pars compacta

We observed that TH-positive cells in the SNpc labeled intensely for the common region of NMDAR1 as well as the C2' splice segment; little staining was observed for the other splice segments (i.e. N1, C1 and C2 segments). Thus, the predominant isoform of NMDAR1 present in these neurons appears to be NR1₀₀₀ (using the terminology of Zukin and Bennett⁵¹). In general, these immunohistochemical data closely parallel results obtained with *in situ* hybridization studies of NMDAR1 splice variant mRNA in the SNpc.^{27,43} That is, the mRNA for the common region of NMDAR1 and isoforms encoding the C2' splice segment are abundant, while isoforms encoding the N1 and C1 segments are scarce. However, some discrepancy appears to exist between the distribution of C2 segment immunoreactivity and the localization of its mRNA; moderate levels of C2 mRNA were reported in the SNpc,⁴³ although we were unable to detect C2 immunoreactivity in these cells. This may be the result of a variety of factors, including the rate of protein translation, turnover of each species and variable intracellular disposition of the protein.⁸

Fig. 4. AMPA receptor immunoreactivity in the SNpc at low magnification. The right panel illustrates immunohistochemical staining for GluR1 (B), GluR2/3 (D) and GluR4 (F). The left panel (A, C, E) illustrates staining of TH in the same sections as B, D and F, respectively. The staining for GluR1 was confined mainly to TH-positive cells in the ventral tier of the SNpc (B). GluR2/3 was more homogeneously distributed throughout the SNpc (D). There was no GluR4 immunoreactivity in the SNpc (F). Scale bar = 200 μ m.



Immunoreactivity for both NMDAR2A and NMDAR2B was weak within TH-positive cells. *In situ* hybridization studies have reported only very low levels of mRNA for NMDAR2B; NMDAR2A mRNA was not detected in the SNpc.⁴³ The only previous study of NMDAR2 subunits is that of Petralia *et al.*³⁷ Using an antibody that recognized both NMDAR2A and NMDAR2B, they found moderate staining in the region of the substantia nigra. Localization within the SNpc was not described, and it is possible that the staining they observed was associated with non-dopaminergic cells.

We have previously demonstrated the presence of mRNAs for two additional NMDA receptor subunits in the SNpc: NMDAR2C and NMDAR2D.⁴³ This abundant expression of NMDAR2D in SNpc is of particular interest because of the unusual physiological properties of channels which contain this subunit. These channels are characterized by an extremely long offset decay and weak Mg²⁺ blockade.³¹ Receptor assemblies containing NMDAR2D also exhibit differential pharmacological properties as compared to channels which contain NMDAR2A or NMDAR2B.²⁶ When well-characterized antibodies for these two subunits become available, it will be of great interest to study the localization of these proteins in TH-positive cells.

Our present analysis of GluR1–GluR4 subunits in the SNpc revealed differential distributions of GluR expression within TH-positive cells. *In situ* hybridization studies suggest that moderate levels of GluR1 mRNA are present.³⁹ Moreover, previous immunohistochemical studies have demonstrated moderate to high levels of the protein in the rat SNpc.^{29,38} Our more detailed confocal microscopic study shows that GluR1 immunoreactivity is distributed heterogeneously; it is most abundant in TH-positive neurons in the ventral tier of the SNpc and scarce in most neurons in the more dorsal part. A similar gradient of GluR1 immunostaining in the SNpc was reported by Paquet *et al.*³⁶ in the squirrel monkey. Other than the dorsal location, the TH-positive neurons lightly stained for GluR1 in our study did not display any morphological features that might differentiate them from the TH-positive neurons which were more strongly labeled for GluR1. *In situ* hybridization studies have shown that GluR2 mRNA is present in high levels in the SNpc, whereas GluR3 mRNA is expressed at relatively low levels in this region.³⁹ We found that GluR2/3 immunostaining was enriched

throughout the SNpc in a homogeneous manner; nearly all TH-positive cells were labeled intensely. These findings are consistent with earlier reports.^{29,36,38} We did not observe any GluR4 immunostaining in TH-positive cells, despite intense labeling of TH-negative cells in the lateral parts of the pars reticulata. The absence of GluR4 immunostaining in the SNpc is in agreement with the earlier immunoperoxidase and dual-label fluorescence study of Martin *et al.*²⁹ However, somewhat different results have been reported by others. Using immunoperoxidase, Paquet *et al.*³⁶ found GluR4 immunoreactivity in the SNpc of the squirrel monkey. Petralia and Wenthold,³⁸ using the same antibody employed in our study, described staining in the region of the rat SNpc, and GluR4 mRNA has been reported to be expressed at moderate levels in the region of the SNpc,³⁹ but it is possible that these results represent expression of GluR4 in adjacent, non-dopaminergic cells or fibers. It might be of value to examine in more detail the localization of GluR4 mRNA in dopaminergic cells of the rat SNpc using dual-label techniques.

Role of N-methyl-D-aspartate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate subunits in regulating nigrostriatal function

In awake animals, dopamine (DA) neurons exhibit both rapid bursting activity, as well as irregular single spikes. *In vitro*, bursting activity is not observed spontaneously, but can be induced by application of NMDA.²² *In vivo*, application of NMDA or AMPA produces dramatic increases in the burst firing patterns of DA neurons, while only NMDA receptor antagonists reduce the rate of firing below baseline.^{9,10,35} In contrast, antagonists of non-NMDA glutamate receptors do not alter the pattern of basal activity of DA neurons and are not as effective at blocking the effects of glutamate on DA neuron firing patterns.^{9,10} Since bursting activity has been correlated with increased release of DA,¹⁵ these data suggest that tonic glutamatergic input mediated by NMDA receptors in the SNpc is responsible for regulating the basal secretion of DA, while both NMDA and AMPA receptors can augment release.

The subunit composition of NMDA receptors is believed to be an important determinant of the physiological and pharmacological properties of the channels (for review see Refs 19 and 51). Our data

Fig. 5. AMPA receptor immunoreactivity in the SNpc at higher magnifications. In each panel, staining of TH-positive cells is shown in green, while immunoreactivity for AMPA receptor subunits is in red. Specific AMPA subunit immunoreactivity is as follows: GluR1 (A, B); GluR2/3 (C, D); GluR4 (E, F). The images in the left panels (A, C, E) are taken at $\times 40$, while the images in the right panels (B, D, F) are taken at $\times 100$. Some of the TH-positive neurons in the SNpc were lightly labeled for GluR1, while others were strongly labeled by GluR1 (A, B). GluR2/3 immunoreactivity was intense in nearly all TH-positive cells (C, D). GluR4 immunostaining was absent from TH-positive cells (E, F). Scale bars = 50 μ m (A, C, E), 10 μ m (B, D, F).

suggest that the predominant form of NMDAR1 in TH-positive cells of the rat SNpc is NR1₀₀₀. Forms lacking the N1 segment are relatively pH insensitive⁴⁷ and have greater sensitivity to glutamate.¹⁷ The C1 cassette contains sites for phosphorylation by protein kinases A and C, as well as a high-affinity binding site for calmodulin;^{12,46} the absence of this segment suggests that SNpc NMDA channels are not strongly regulated by these mechanisms. SNpc neurons do, however, appear to accumulate NMDAR1 splice form containing the C2' terminus, which has a consensus sequence for interaction with the PDZ domains of postsynaptic density protein-95.²⁵ This postsynaptic density protein interacts with NMDAR2 subunits and other proteins, including neuronal nitric oxide synthase, and participates in clustering of receptor subunits at synaptic sites.^{6,23,24} The NMDAR2 subunits also make important contributions to the properties of NMDA channels, particularly with respect to the affinities of agonists and antagonists.^{26,28,30} Our data suggest that there is little NMDAR2A or NMDAR2B present in SNpc neurons, although these subunits are found in neurons in many other brain structures. If the predominance of NMDAR2C and NMDAR2D predicted by *in situ* hybridization studies⁴³ is correct, then it is likely that the receptor properties of SNpc NMDA receptors will differ substantially from NMDA receptors in most other regions.

Our data suggest that there is also selective expression of AMPA subunits within the SNpc, both with respect to other regions of the brain and within the SNpc itself. We found immunostaining for GluR2/3 in all of the TH-positive neurons of the SNpc. Based on the earlier *in situ* hybridization studies,³⁹ it seems likely that this largely reflects the presence of GluR2 subunits, which have an important effect on the calcium permeability of AMPA channels.^{18,48} We also found that, in the rat SNpc, GluR1 immunoreactivity is abundant in the neurons in the ventral

part of the SNpc, but scarce in the dorsal part. This is of interest because it parallels the pattern of neuronal vulnerability observed in PD, where the neuronal loss is most severe in the ventrolateral region of the SNpc.¹³ Extending our knowledge of the anatomical distribution of glutamate receptors in the SNpc will provide important insight into the pathophysiological properties of these receptors and the subsequent development of therapeutic agents to treat PD.

CONCLUSIONS

Using a well-characterized panel of antibodies to NMDA and AMPA receptor subunits and dual-label confocal microscopy, we have found that the dopaminergic neurons of the rat SNpc contain the NMDAR1 receptor subunit, but little NMDAR2A or NMDAR2B protein. The predominant isoform of NMDAR1 appears to be the form lacking all three variably spliced segments (N1, C1 and C2; the NR1₀₀₀ isoform) and having instead the alternative C2' terminus. Staining for GluR2/3 was found in all the TH-positive neurons of the SNpc, but staining for GluR1 was heterogeneous, with intense immunoreactivity concentrated in the ventral tier of the SNpc. There was no observable GluR4 immunoreactivity in the SNpc. The receptor subunits present are likely to make important contributions to both the normal physiological regulation of DA neuron activity, as well as the vulnerability of these cells to disease processes.

Acknowledgements—This work was supported by United States Public Health Service Grants NS31579 and NS34361. Dr Standaert is a recipient of a Cotzias Fellowship from the American Parkinson Disease Association. We would like to thank Dr Morgan Sheng of the Neurobiology Department and Howard Hughes Medical Institute at the Massachusetts General Hospital, Dr Ted Dawson from the Johns Hopkins University and Dr Robert Wenthold from the NINDS/NIH for providing some of the antibodies used in this study.

REFERENCES

1. Albin R. L., Makowiec R. L., Hollingsworth Z. R., Dure L. S., Penney J. B. and Young A. B. (1992) Excitatory amino acid binding sites in the basal ganglia of the rat: a quantitative autoradiographic study. *Neuroscience* **46**, 35–48.
2. Aoki C., Rhee J., Lubin M. and Dawson T. M. (1997) NMDA-R1 subunit of the cerebral cortex co-localizes with neuronal nitric oxide synthase at pre- and postsynaptic sites and in spines. *Brain Res.* **750**, 25–40.
3. Aoki C., Venkatesan C., Go C.-C., Mong J. A. and Dawson T. M. (1994) Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. *J. Neurosci.* **14**, 5202–5221.
4. Beal M. F. (1992) Mechanisms of excitotoxicity in neurologic diseases. *Fedn Proc. Fedn Am. Socs exp. Biol.* **6**, 3338–3344.
5. Blandini F., Porter R. H. P. and Greenamyre J. T. (1996) Glutamate and Parkinson's disease. *Molec. Neurobiol.* **12**, 73–94.
6. Brenman J. E., Chao D. S., Gee S. H., McGee A., Craven S. E., Santillano D. R., Huang F., Xia H., Peters M. F., Froehner S. C. and Brecht D. S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha 1-syntrophin mediated by PDZ domains. *Cell* **84**, 757–767.
7. Burnashev N., Monyer H., Seeburg P. H. and Sakmann B. (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8**, 189–198.
8. Chazot P. L. and Stephenson F. A. (1997) Biochemical evidence for the existence of a pool of unassembled C2 exon-containing NR1 subunits of the mammalian forebrain NMDA receptor. *J. Neurochem.* **68**, 507–516.
9. Chergui K., Charléty J., Akaoka H., Saunier C. F., Brunet J.-L., Buda M., Scensson T. H. and Chouvet G. (1993) Tonic activation of NMDA receptors causes spontaneous burst discharge of rat midbrain dopamine neurons *in vivo*. *Eur. J. Neurosci.* **5**, 137–144.

10. Christoffersen C. L. and Meltzer L. T. (1995) Evidence for *N*-methyl-D-aspartate and AMPA subtypes of the glutamate receptor on substantia nigra dopamine neurons: possible preferential role for *N*-methyl-D-aspartate receptors. *Neuroscience* **67**, 373–381.
11. Durand G. M., Bennett M. V. L. and Zukin R. S. (1993) Splice variants of the *N*-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. *Proc. natn. Acad. Sci. U.S.A.* **90**, 6731–6735.
12. Ehlers M. D., Zhang S., Bernhardt J. P. and Huganir R. L. (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**, 745–755.
13. Fearnley J. and Lees A. (1991) Aging and Parkinson's disease; substantia nigra regional selectivity. *Brain* **114**, 2283–2301.
14. Follesa P. and Ticku M. K. (1996) Chronic ethanol-mediated up-regulation of the *N*-methyl-D-aspartate receptor polypeptide subunits in mouse cortical neurons in culture. *J. biol. Chem.* **271**, 13,297–13,299.
15. Gonon F. G. (1988) Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by *in vivo* electrochemistry. *Neuroscience* **24**, 19–28.
16. Gracy K. N. and Pickel V. M. (1995) Comparative ultrastructural localization of the NMDAR1 glutamate receptor in the rat basolateral amygdala and bed nucleus of the stria terminalis. *J. comp. Neurol.* **362**, 71–85.
17. Hollmann M., Boulter J., Maron C., Beasley L., Sullivan J., Pecht G. and Heinemann S. (1993) Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron* **10**, 943–954.
18. Hollmann M., Hartley M. and Heinemann S. (1991) Ca²⁺ permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* **249**, 56–60.
19. Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *A. Rev. Neurosci.* **17**, 31–108.
20. Iadarola M. J., Kim D. J. and Caudle R. M. (1996) Analysis of C-terminal splice variant of the NMDA NR1 subunit in spinal cord using sequence specific antisera. *Soc. Neurosci. Abstr.* **22**, 1996.
21. Ishii T., Moriyoshi K., Sugihara H., Sakurada K., Kadotani H., Yokoi M., Akazawa C., Shigemoto R., Mizuno N., Masu M. and Nakanishi S. (1993) Molecular characterization of the family of *N*-methyl-D-aspartate receptor subunits. *J. biol. Chem.* **268**, 2836–2843.
22. Johnson S. W., Seutin V. and North R. A. (1992) Burst firing in dopamine neurons induced by *N*-methyl-D-aspartate: role of the electrogenic sodium pump. *Science* **258**, 665–667.
23. Kennedy M. B. (1997) The postsynaptic density at glutamatergic synapses. *Trends Neurosci.* **20**, 264–268.
24. Kim E., Cho K.-O., Rothschild A. and Sheng M. (1996) Heteromultimerization and NMDA receptor-clustering activity of chapsyn-110, a member of the PSD-95 family of proteins. *Neuron* **17**, 103–113.
25. Kornau H. C., Schenker L. T., Kennedy M. B. and Seeburg P. H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**, 1737–1740.
26. Laurie D. J. and Seeburg P. H. (1994) Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. *Eur. J. Pharmac.* **268**, 335–345.
27. Laurie D. J. and Seeburg P. H. (1994) Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. *J. Neurosci.* **14**, 3180–3194.
28. Lynch D. R., Anegawa N. J., Verdoorn T. and Pritchett D. B. (1994) *N*-Methyl-D-aspartate receptors—different subunit requirements for binding of glutamate antagonists, glycine antagonists, and channel-blocking agents. *Molec. Pharmac.* **45**, 540–545.
29. Martin L. J., Blackstone C. D., Levey A. I., Huganir R. L. and Price D. L. (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. *Neuroscience* **53**, 327–358.
30. Monaghan D. T. and Larsen H. (1997) NR1 and NR2 subunit contributions to *N*-methyl-D-aspartate receptor channel blocker pharmacology. *J. Pharmac. exp. Ther.* **280**, 614–620.
31. Monyer H., Burnashev N., Laurie D. J., Sakmann B. and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**, 529–540.
32. Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B. and Seeburg P. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* **256**, 1217–1221.
33. Moriyoshi K., Masu M., Ishii T., Shigemoto R., Mizuno N. and Nakanishi S. (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**, 31–37.
34. Nakanishi N., Axel R. and Schneider N. A. (1992) Alternative splicing generates functionally distinct *N*-methyl-D-aspartate receptors. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8552–8556.
35. Overton P. and Clark D. (1992) Iontophoretically administered drugs acting at the *N*-methyl-D-aspartate receptor modulate burst firing in A9 dopamine neurons in the rat. *Synapse* **10**, 131–140.
36. Paquet M., Tremblay M., Soghomonian J.-J. and Smith Y. (1997) AMPA and NMDA glutamate receptor subunits in midbrain dopaminergic neurons in the squirrel monkey: an immunohistochemical and *in situ* hybridization study. *J. Neurosci.* **17**, 1377–1396.
37. Petralia R. S., Wang Y.-X. and Wenthold R. J. (1994) The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. *J. Neurosci.* **14**, 6102–6120.
38. Petralia R. S. and Wenthold R. J. (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J. comp. Neurol.* **318**, 329–354.
39. Sato K., Kiyama H. and Tohyama M. (1993) The differential expression patterns of messenger RNAs encoding non-*N*-methyl-D-aspartate glutamate receptor subunits (GluR1–4) in the rat brain. *Neuroscience* **52**, 515–539.
40. Sheng M., Cummings J., Roldan L. A., Jan Y. N. and Jan L. Y. (1994) Changing subunit composition of heteromeric NMDA receptors during development. *Nature* **368**, 144–147.
41. Siegel S. J., Brose N., Janssen W. G., Gasic G. P., Jahn R., Heinemann S. F. and Morrison J. H. (1994) Regional, cellular, and ultrastructural distribution of *N*-methyl-D-aspartate receptor subunit 1 in monkey hippocampus. *Proc. natn. Acad. Sci. U.S.A.* **91**, 564–568.
42. Standaert D. G., Needleman P. and Saper C. B. (1986) Organization of atropine-like immunoreactive neurons in the central nervous system of the rat. *J. comp. Neurol.* **253**, 315–341.
43. Standaert D. G., Testa C. M., Penney J. B. and Young A. B. (1994) Organization of *N*-methyl-D-aspartate glutamate receptor gene expression in the basal ganglia of the rat. *J. comp. Neurol.* **343**, 1–16.

44. Sugihara H., Moriyoshi K., Ishii T., Masu M. and Nakanishi S. (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem. biophys. Res. Commun.* **185**, 826–832.
45. Testa C. M., Friberg I. K., Weiss S. W. and Standaert D. G. (1998) Immunohistochemical localization of metabotropic glutamate receptors mGluR1a and mGluR2/3 in the rat basal ganglia. *J. comp. Neurol.* **390**, 5–19.
46. Tingley W. G., Roche K. W., Thompson A. K. and Huganir R. L. (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* **364**, 70–73.
47. Traynelis S. F., Hartley M. and Heinemann S. F. (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science* **268**, 873–876.
48. Verdoorn T. A., Burnashev N., Monyer H., Seeburg P. H. and Sakmann B. (1991) Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* **252**, 715–718.
49. Weiss S. W., Albers D. S., Iadarola M. J., Dawson T. M., Dawson V. L. and Standaert D. G. (1998) NMDAR1 glutamate receptor subunit isoforms in neostriatal, neocortical and hippocampal nitric oxide synthase neurons. *J. Neurosci.* **18**, 1725–1734.
50. Wenthold R. J., Yokotani N., Doi K. and Wada K. (1992) Immunohistochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies: evidence for a hetero-oligomeric structure in rat brain. *J. Biol. Chem.* **267**, 501–507.
51. Zukin R. S. and Bennett M. V. L. (1995) Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci.* **18**, 306–313.

(Accepted 26 May 1998)