

CHOLINERGIC NEURONS OF THE ADULT RAT STRIATUM ARE IMMUNOREACTIVE FOR GLUTAMATERGIC N-METHYL-D-ASPARTATE 2D BUT NOT N-METHYL-D-ASPARTATE 2C RECEPTOR SUBUNITS

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Abstract—Cholinergic neurons of the striatum play a crucial role in controlling output from this region. Their firing is under the control of a relatively limited glutamatergic input, deriving principally from the thalamus. Glutamate transmission is effected via three major subtypes of receptors, including those with affinity for *N*-methyl-*D*-aspartate (NMDA) and the properties of individual receptors reflect their precise subunit composition. We examined the distribution of NMDA2C and NMDA2D subunits in the rat striatum using immunocytochemistry and show that a population of large neurons is strongly immunoreactive for NMDA2D subunits. From their morphology and ultrastructure, these neurons were presumed to be cholinergic and this was confirmed with double immunofluorescence. We also show that NMDA2C is present in a small number of septal and olfactory cortical neurons but absent from the striatum.

Receptors that include NMDA2D subunits are relatively insensitive to magnesium ion block making neurons more likely to fire at more negative membrane potentials. Their localization to cholinergic neurons may enable very precise regulation of firing of these neurons by relatively small glutamatergic inputs. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: interneuron, ultrastructure, synapse, endoplasmic reticulum.

The cholinergic neurons of the striatum play an important role in basal ganglia control of voluntary movement. They represent only 1–2% of striatal neurons but their extensive local axon collateral system, innervating both medium-sized densely spiny neurons (MSN) (Izzo and Bolam, 1988) and other local circuit neurons (Koos and Tepper, 2002) is consistent with a primary role in determining the final activity of striatal output neurons (Calabresi et al., 2000). While the majority of glutamatergic input to MSN originates in the cortical regions, excitatory input to these tonically active, cholinergic striatal neurons comes almost exclusively from the parafascicular thalamic nucleus (Lapper and Bolam, 1992; Zhou et al., 2002) and possibly from

the cortex (Thomas et al., 2000), and principally from the midline/intralaminar thalamic nuclei to the ventral striatum (Meredith and Wouterlood, 1990).

The firing of cholinergic neurons is under the control of a relatively few inputs and interactions are subserved by a number of different glutamate receptor subtypes. Although no AMPA subunits have been reported (Chen et al., 1996; Fujiyama et al., 2004), cholinergic neurons express kainate GluR5, 6 and 7 subunits (Chen et al., 1996). Moreover, stimulation of metabotropic receptor subunits, mGluR1-3, 5 and 7 (Bell et al., 2002) exerts a profound effect on cell excitability (Di Chiara et al., 1994). In addition, *N*-methyl-*D*-aspartate (NMDA) agonists potentiate striatal acetylcholine release (Giovannini et al., 1995) while direct stimulation of the thalamostriatal pathway increases acetylcholine release, via action on *N*-methyl-*D*-aspartate receptors (NR) (Consolo et al., 1996). Double labeling studies combining *in situ* hybridization for subunit mRNA with immunolabeling with antibodies to choline acetyl transferase, show that cholinergic interneurons have abundant NR subunit 1, which is present in all functional NRs, along with NR2B and NR2D subunits (Landwehrmeyer et al., 1995; Standaert et al., 1996, 1999; Kuppenbender et al., 2000).

The identity of the specific NR2 subunit is particularly important when defining the pharmacological and biophysical properties of NRs. In particular, receptors containing NR1/2C and NR1/2D receptor subunits show a more rapid response to fast depolarizations (Clarke and Johnson, 2006) making them less effective as coincidence detectors, and they are less sensitive to blockade by magnesium ions (Momiya et al., 1996) allowing for stronger activity at relatively negative membrane potentials. Moreover, the presence of NR2D confers a greater affinity for glutamate (Kuppenbender et al., 2000). To further our understanding of the glutamatergic control of striatal cholinergic neurons, we set out to demonstrate whether they express NR2C or NR2D receptor subunit protein.

EXPERIMENTAL PROCEDURES

All animal experiments were conducted in compliance with the Home Office Guidance under the UK Animals (Scientific Procedures) Act 1986, associate guidelines and European directive (86/609EEC). The work was designed to respect animal welfare, to minimize suffering and the number of animals used.

Four male Lister hooded rats were perfused with a mixture of paraformaldehyde (4%) and glutaraldehyde (0.1%) in phosphate buffer (0.1 M, pH 7.4). Their brains were removed and sectioned using a vibrating microtome (Leica, Milton Keynes, UK). Sections

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Abbreviations: ChAT, choline acetyltransferase; MSN, medium-sized densely spiny neurons; NMDA, *N*-methyl-*D*-aspartate; NR, *N*-methyl-*D*-aspartate receptor; PBS, 0.01 M phosphate-buffered saline.

were collected in four parallel series from about 3.5 mm rostral to about 1 mm caudal to Bregma to include both dorsal and ventral striatum. Three series of sections were used in this study.

To enhance antibody penetration, the tissue was irradiated with microwaves, a technique successfully used with other NMDA subunits (Fritschy et al., 1998). Briefly, sections were incubated overnight at room temperature in sodium citrate solution, composed of 0.1 M citric acid and 0.2 M Na₂HPO₄ (pH 4.5). They were then transferred into 80 ml fresh buffer and irradiated in a domestic microwave oven at 650 W for 40 s. The tissue was then cooled to approximately 40 °C and transferred into 0.01 M phosphate-buffered saline (PBS), pH 7.4.

Prior to immunohistochemistry, sections were incubated for 5 min in sodium borohydride (0.5% in PBS), washed in PBS until all bubbles had been removed, incubated with normal donkey serum (10% in PBS) for 30 min and treated with Triton X-100 (0.1% in PBS) for a further 30 min. Sections for electron microscopy were exposed neither to sodium borohydride nor to Triton X-100.

Single immunolabeling for NR2 subunits

For single labeling, two series of sections were selected, one was incubated with goat anti-NR2C subunit and the other with goat anti-NR2D subunit antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 and 1:500 respectively. The NR2C antibody is raised to the C-terminal peptide of the mouse protein and in Western blots recognizes a single band (Chan et al., 2002). The NR2D antibody has also been characterized using Western blots (Chan et al., 2002), and a number of studies show that it does not cross-react with NR1 or any other NR2 subunits (Hollmann and Heinemann, 1994; Watanabe et al., 1994; Glover et al., 2000). These antibodies have previously been used to label NR2C and NR2D subunits in optic nerve (Salter and Fern, 2005) and in rat brain (Lindahl and Keifer, 2004). Control sections were not exposed to the primary antibody but were processed otherwise identically to experimental sections.

Antibody binding was amplified by incubation in biotinylated donkey anti-goat (1:200 in PBS, Stratech, Soham, UK) for 1 h then 1:100 avidin–biotin–peroxidase complex (Vector Laboratories, Peterborough, UK). The bound peroxidase was revealed by incubating in 0.05% diaminobenzidine tetrahydrochloride in 0.05 M Tris–HCl buffer with 0.01% hydrogen peroxide for 10 min. The reaction was terminated with excess buffer. Sections for light microscopy were rinsed in PBS and transferred to gelatin-coated slides, air-dried, dehydrated and mounted using XAM (Merck, Nottingham, UK).

Double immunolabeling for NR2D and choline acetyltransferase (ChAT)

Sections from a third series, selected to include the striatum at the level of the decussation of the corpus callosum and caudal to this, were processed for double immunolabeling. They first underwent the microwave irradiation and pretreatments detailed above. Sections were then incubated sequentially in antibodies for ChAT and NR2D. The sections were incubated in rabbit anti-ChAT primary antibody (Chemicon, Chandlers Ford, UK) at a dilution of 1:500 with 0.1% Triton-X, overnight at 4 °C. They were then washed in PBS and incubated in 1:1000 goat anti-rabbit Alexafluor 488 (Molecular Probes, Paisley, UK), overnight at 4 °C. After extensive washes in PBS, sections were then processed for NR2D immunohistochemistry, using the antibody described above, followed by incubation in donkey anti-goat Alexafluor 594 (Molecular Probes) overnight. Finally, they were transferred to gelatin-coated slides and mounted using Vectashield permanent mounting medium (Vector Laboratories).

Electron microscopy

Following immunolabeling for NR2D subunits, some sections from each animal were selected for electron microscopy. These were rinsed in 0.1 M phosphate buffer, flattened onto watch glasses and immersed in 1% osmium tetroxide in phosphate buffer for 30 min. After a brief wash in water, they were dehydrated through a series of alcohols, with 1% uranyl acetate at the 70% stage to provide extra contrast to the tissue. After 100% ethanol, sections were placed in propylene oxide for 20 min and then transferred to Durcupan resin (Sigma-Aldrich, Gillingham, Dorset), overnight at room temperature. Once the resin had penetrated the sections, they were mounted on slides in the same resin and cured for 48 h at 60 °C. Regions of interest in the dorsal striatum were identified in the light microscope re-embedded, re-sectioned at 60 nm and serial sections collected onto pioloform-coated, single slot, copper grids. After further contrast enhancement (Reynolds, 1963), they were examined in a Philips 410 electron microscope.

A semi-quantitative study was carried out. Images were taken of every immunolabeled profile encountered in a systematic sweep of a single section at a magnification of $\times 6400$. This was carried out for sections from four blocks, prepared from three different rats. All blocks were prepared from dorsal striatum, from sections at around the level of Bregma. The total area examined was measured from the surface of the trimmed block and summed.

Image preparation

Images were prepared either on conventional film, printed and scanned (light micrographs) or acquired digitally (light, fluorescent and electron micrographs). In double-labeled tissue, both dorsal and ventral striatum were examined, using a Leica fluorescent microscope with the appropriate filters, for co-localization of ChAT and NMDA 2D immunoreactive structures. Images containing representative examples of labeling with both antibodies were acquired digitally using a Leica DFC300 FX digital fire-wire-cooled camera running DFC Twain and Image Manager (Leica). All images were imported into Adobe Photoshop, cropped, adjusted for contrast and in some cases color balance, and assembled into plates and labels were applied.

RESULTS

Immunohistochemistry: light microscopic level

Specific immunostaining for NR2C was absent from both dorsal and ventral striatum, although levels of background staining were quite high. This diffuse background brown coloration was restricted to superficial levels and had no association to any neuronal or glial profiles. Control sections showed no staining. However, in experimental tissue, rostrally, a small but densely stained population of medium-to-large (15–25 μm) neurons formed an arc along the medio-ventral edge of the adjacent olfactory cortex and taenia tectum (Fig. 1A, B, C). Although some of these neurons had prominent apical dendrites (Fig. 1B2, B3), in others it was possible to see indentations in the nuclei. In addition, in the dorsal septum, groups of medium to large multipolar cells were strongly immunopositive (Fig. 1D).

Neurons immunopositive for NR2D subunits were evenly distributed across the striatal complex, from the most rostral extent of the nucleus accumbens, to the most caudal level examined (about 1 mm caudal to Bregma) in both the ventral (Fig. 2A) and dorsal (Fig. 2B–D) striatum. In the ventral striatum cell bodies were between 20 and

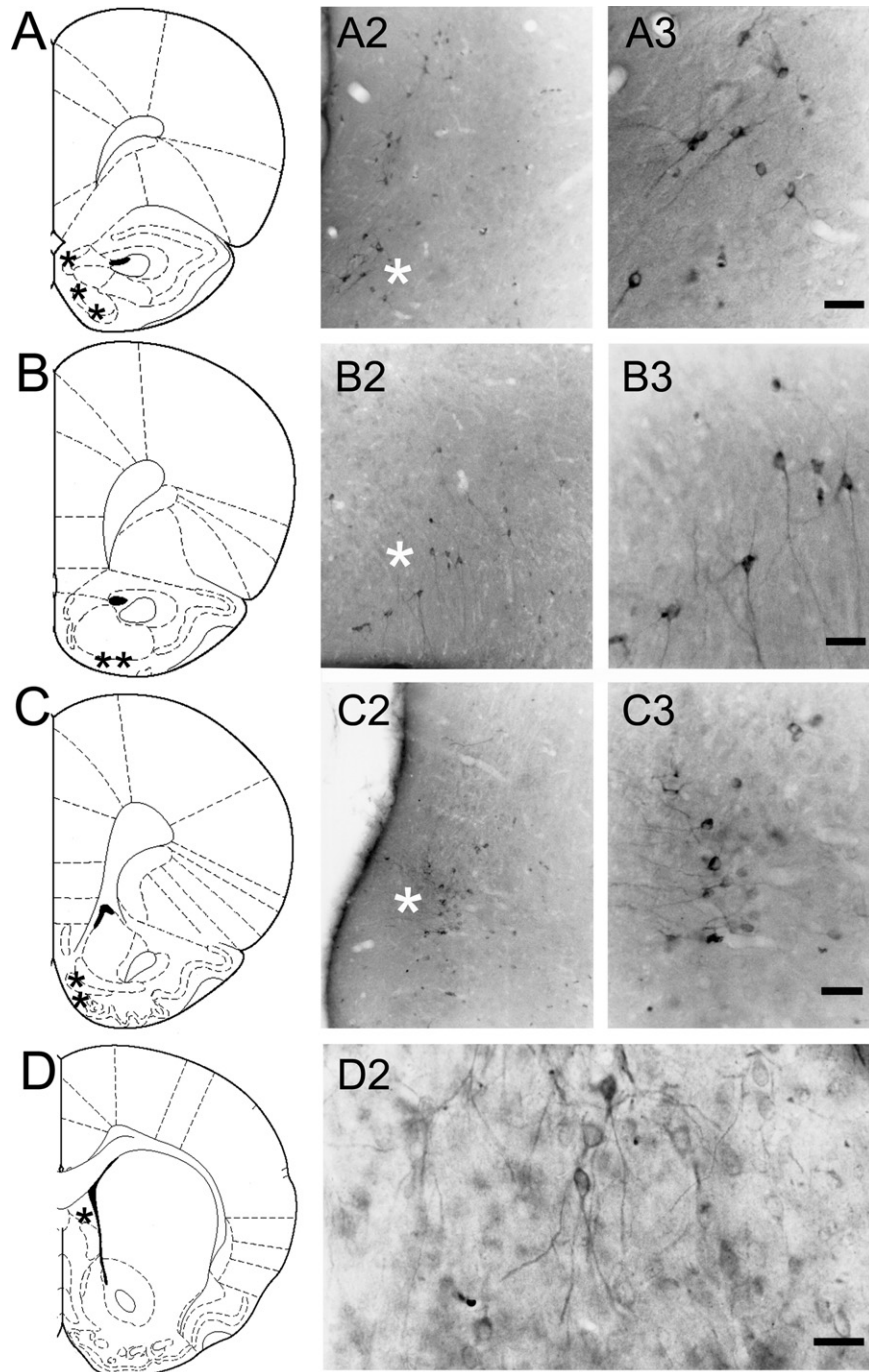


Fig. 1. NR2C subunit immunolabeling. (A) Medium-sized to large neurons in the rostro-medial olfactory cortex/taenia tectum are sparsely distributed (A2) and lack any obvious orientation to their dendrites (A3). (B) Pyramidal-like neurons in the ventral olfactory cortex, many with apical dendrites oriented ventrally (B2) and oval to pyramidal-shaped somata (B3). (C) Medium-sized to large neurons more caudally in the olfactory cortex are sparsely distributed and lack obvious orientation (C2, C3). (D) Large, multipolar neurons in the laterodorsal septum have oval cell bodies and long dendrites that branch infrequently (D2). White asterisks in two indicate the regions shown at higher magnification in 3. Scale bars=50 μm .

30 μm in diameter, whereas in the dorsal striatum they were larger (25–40 μm). In both brain regions, immunopositive cells gave rise to two or three long, smooth dendrites which seldom branched (Fig. 2A, B, D). At the light microscopic level, nuclei were seen to be eccentric in the abundant cytoplasm (Fig. 2B–D) and nuclear indentations were

sometimes visible (Fig. 2C, D). Labeling extended several hundred microns from the cell body, into secondary and tertiary dendrites. In addition, there was a population of immunopositive cells that had slightly smaller (15–20 μm) somata and bipolar dendrites (Fig. 2C, D). Pre-treatment with sodium borohydride reduced the level of background

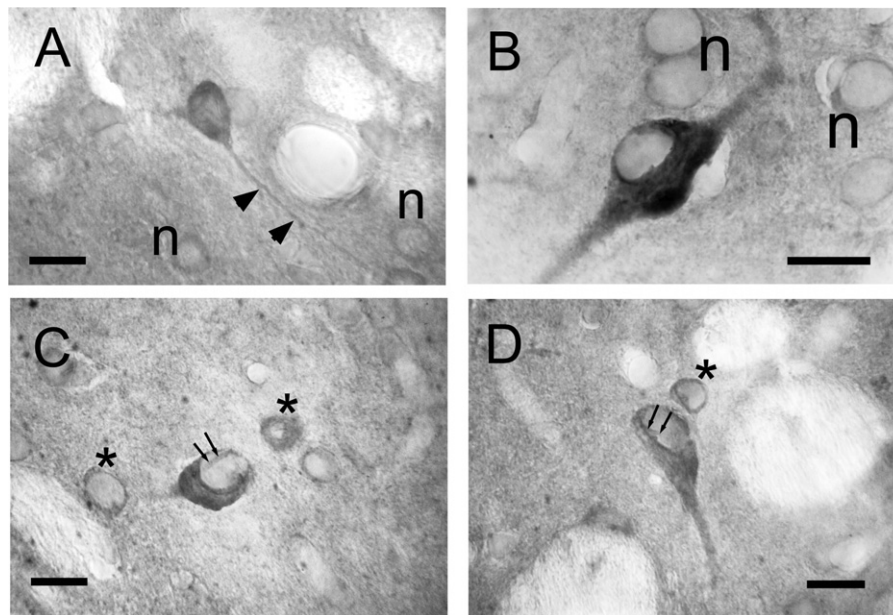


Fig. 2. NR2D subunit immunolabeling. (A) A large, multipolar immunoreactive neuron in the ventral striatum has long, smooth dendrites that seldom branch. Note smaller somata (n) with sparse cytoplasm and smooth nuclei are also lightly labeled. (B) An immunoreactive neuron in the dorsal striatum is larger than that shown in A and has large smooth dendrites and an eccentrically located nucleus. Note again the adjacent smaller neurons (n) with sparse cytoplasm and smooth nuclei. (C, D) The somata of large immunopositive neurons in the dorsal striatum have eccentric, deeply indented nuclei (small arrows) and abundant cytoplasm. Adjacent smaller, lightly labeled neurons (asterisks) also have indented nuclei. Scale bars A, C, D, 25 μm ; B, 20 μm .

staining but in all sections medium-sized somata with smooth round nuclei and sparse cytoplasm were apparently lightly labeled (Fig. 2A, B).

Immunofluorescence and double labeling

In tissue labeled using immunofluorescence, the morphology of NR2D immunopositive neurons resembled those labeled using the DAB histochemistry (Figs. 2, 3). In the same tissue sections, cells in the striatum with the characteristic morphology of cholinergic neurons were labeled with the ChAT antibody (Fig. 3A, B). When comparing labeling with the two antibodies, it was clear that the majority, but not all, of ChAT positive neurons were also immunopositive for NR2D subunits (Fig. 3C).

Immunohistochemistry: electron microscopic level

In tissue prepared for electron microscopic evaluation, labeling was largely restricted to the cell bodies, except in the most superficial levels of the tissue. In the electron microscope, large neuronal somata were immunolabeled, with reaction product deposited on membranes in the cytoplasm, principally the endoplasmic reticulum (Fig. 4A). The majority (70%) of the 57 immunolabeled profiles, collected in a semi-systematic survey of a total of 1.2 mm^2 tissue from four blocks prepared from three rats, were between 0.5–1.0 μm at the minimum diameter. Within this sample, 40% (23/57) of profiles received synaptic input, of which two thirds were symmetrical and one third asymmetrical. Small dendrites (<0.5 μm diameter) were rarely the target of boutons forming symmetrical synaptic specializations but were the commonest target for those forming

asymmetrical synaptic specializations. Immunolabeled dendrites were not evenly distributed within the tissue, presumably being concentrated in regions containing immunolabeled cell bodies. Five strongly immunolabeled neuronal somata were encountered in this sample.

One example of an immunolabeled cell body was examined in a fully correlated study. A large immunolabeled neuron, identified in the light microscope, was found to have abundant cytoplasm and a deeply indented nucleus, with a nuclear rod-like inclusion (Fig. 4A). Immunolabeling was again most obvious along the endoplasmic reticulum. In a single section through the soma, six asymmetric synaptic specializations (Fig. 4B–E) and one symmetric (Fig. 4C) synaptic specialization were identified.

DISCUSSION

This study is the first to examine the distribution of NR2C and NR2D protein in the rat striatal complex at the cellular level. We show that neurons in this brain region do not express NR2C subunits. We also find that NR2D subunits are present in a population of large neurons and show these to be the large cholinergic interneurons. NR2D subunits are also present in some smaller neurons that might equate with the somatostatin-containing population.

This distribution is in broad agreement with studies localizing the mRNA for NR2D subunits. Combining *in situ* hybridization for mRNA coding NR2 subunits with immunolabeling, interneurons, including those containing acetylcholine or somatostatin, were found to express NR2D subunits of the NR (Landwehrmeyer et al., 1995; Standaert

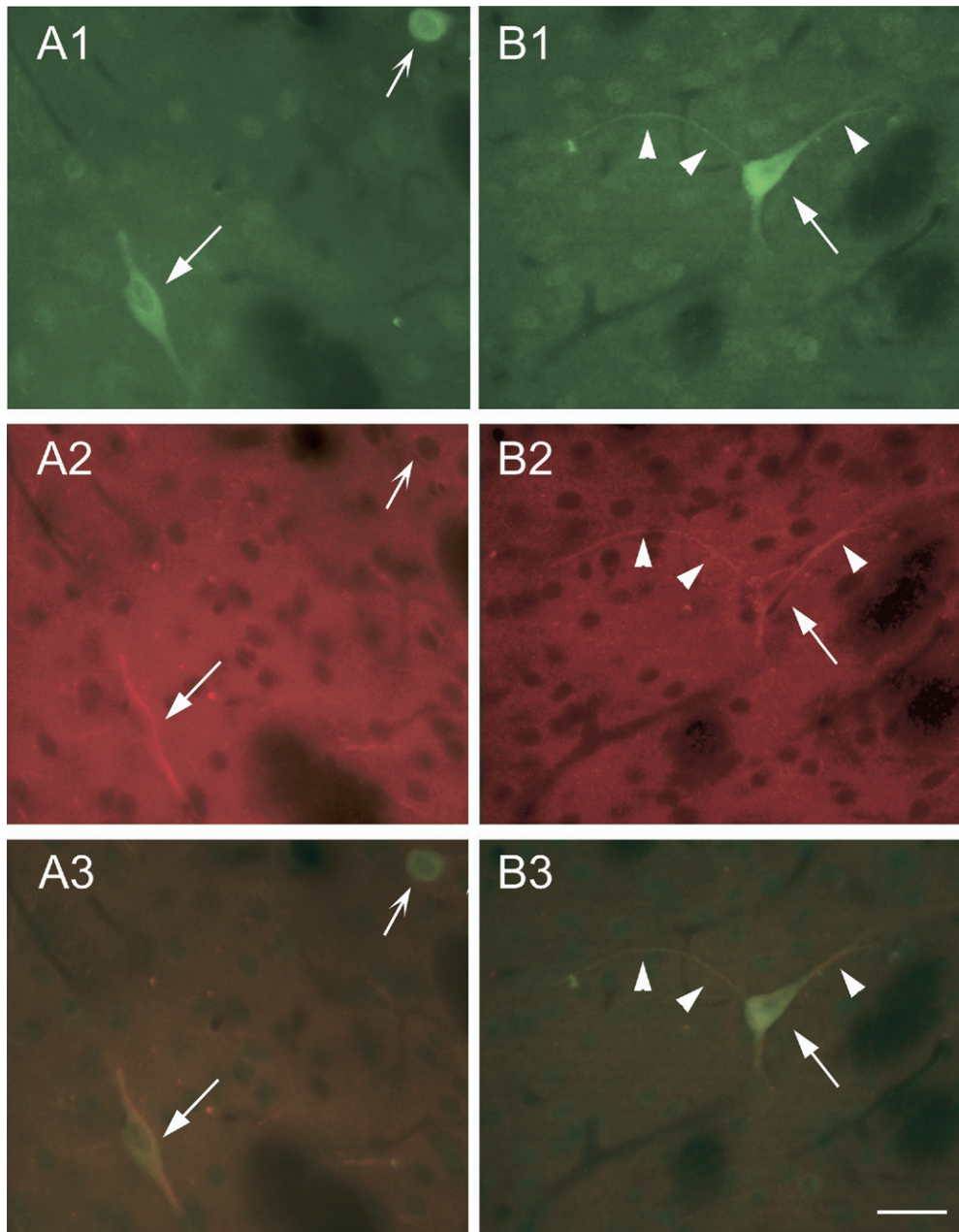


Fig. 3. Some NR2D-immunopositive cells are cholinergic. ChAT immunoreactive cells are visible throughout dorsal and ventral striatum, displaying the typical morphological features of cholinergic neurons, including eccentrically located nuclei (arrow) and local labeling of dendrites (arrowheads) (A1, B1). In the same field, NR2D immunoreactive cells resemble those seen with immunohistochemistry, with labeling of both the soma and proximal dendrites of cells (straight arrows and arrowheads) (A2, B2). The majority of ChAT immunoreactive neurons are also NR2D-immunoreactive (straight arrows), as indicated in the merged images (A3, B3). However, not all ChAT immunoreactive neurons were NR2D immunoreactive (see A1, A2 and A3, where the smaller round soma is ChAT but not NR2D positive, indented arrow). Note also that dendritic labeling for NR2D in the cell in B2 is more extensive than ChAT immunoreactivity in the same cell (B1). Scale bars=30 μm A and B (scale bar in B3).

et al., 1996). The same techniques show, in rat, small amounts of NR2C message in neurons capable of synthesizing GABA but not in cholinergic- or somatostatin-containing populations (Standaert et al., 1999), in contrast to human where cholinergic neurons also code for NR2C subunits (Kuppenbender et al., 2000). It is possible that, in the rat, all MSN also produce low levels of NR2D subunits although there is no evidence that these are integrated into

post-synaptic membranes. No immunolabeled spines were seen.

Immunoreactivity for NR2D subunits was present in cholinergic interneurons. At the light microscopic level, in the dorsal striatum NR2D positive neurons had large somata, and a few long dendrites that seldom branched and were spine-free (Phelps et al., 1985). In the ventral striatum/nucleus accumbens, immunoreactive neurons

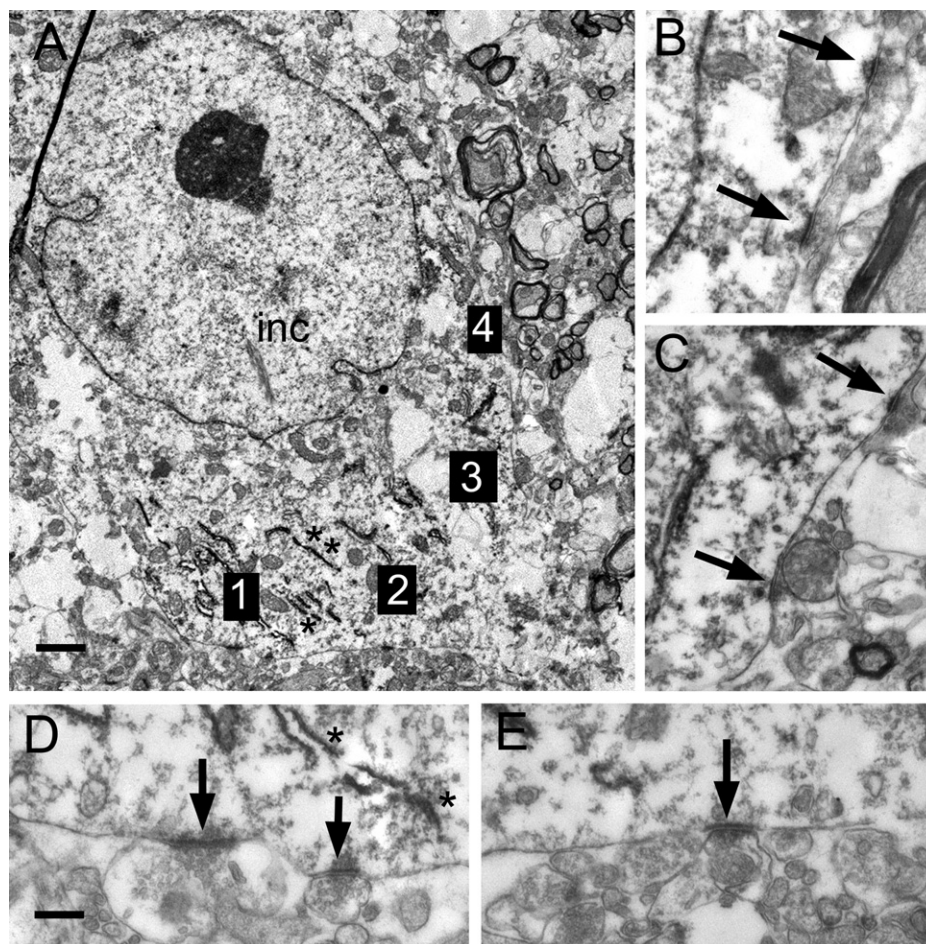


Fig. 4. NR2D immunoreactive neuron. The soma of a large immunoreactive neuron (A) from the dorsal striatum has abundant cytoplasm and an indented nucleus with a rod-like inclusion (inc). Immunoreaction product is associated with the endoplasmic reticulum (asterisks, A, D). Region 1 is shown at higher magnification in D, regions 2 in E, region 3 in C and region 4 in B. Boutons form asymmetrical (B, C upper arrow, D, E) or symmetric (C, lower arrow) synaptic contacts (arrows) with the soma. There is no particular association of immunolabeling with synaptic membranes. Scale bars = 1 μm A, B–E (scale bar in D) 0.25 μm .

were smaller than in the dorsal regions but still matched previous descriptions of cholinergic neurons (Phelps and Vaughn, 1986). Moreover, these neurons were immunopositive for the enzyme ChAT, which is present in cholinergic neurons.

The immunolabeled neurons also display the ultrastructural characteristics of cholinergic interneurons. They were large, with abundant cytoplasm and extensive endoplasmic reticulum. The nucleus was invaginated and, although intranuclear rods are most often associated with striatal GABAergic interneurons (Ribak et al., 1979; Bolam et al., 1984) such nuclear inclusions have been reported in cholinergic neurons (Phelps et al., 1985).

It has also been reported that NR2D subunit mRNA is expressed by somatostatin-containing neurons in the striatum. These can be distinguished from cholinergic neurons by their smaller size (Di Figlia and Aronin, 1982), even in the ventral striatum (Vincent et al., 1985) and we concentrate our study on the larger neurons. In the dual labeling study, most of the large neurons immunoreactive for NR2D subunits were also immunolabeled with ChAT.

Moreover, since somatostatin-containing neurons are not reported to have nuclear inclusions and synaptic inputs to their somata are rare (Vincent and Johansson, 1983) the neuron presented in this study is highly likely to be cholinergic. Nevertheless, we cannot rule out the possibility that some of the smaller labeled profiles seen in both the light and electron microscopic studies are somatostatin- rather than acetylcholine-containing neurons.

Technical issues

Both NR2 subunit antibodies stained discrete, but different, populations of neurons in the rat brain. The NR2C antibody failed to produce any specific striatal neuronal labeling. Nevertheless, neurons with strong immunolabeling were present the olfactory cortex and septum in the same tissue sections, indicating that these neurons may express functionally relevant amounts of the NR2C subunit (Plant et al., 1997). Neurons in these forebrain regions have never previously been reported to express the NR2C subunit. The distribution of NR2C-immunola-

beled neurons appears to coincide with the rostral migratory stream for cells generated in the subventricular zone (Wonders and Anderson, 2006). Nevertheless, NR2C subunits are only expressed post-natally and reach adult levels by 21 days after birth (Wenzel et al., 1997), suggesting that immunolabeled cells are relatively mature, a conclusion supported by their complex dendritic morphology.

The NR2D subunit antibody strongly labels neurons with the morphology of cholinergic interneurons and this was confirmed in double-labeling studies. Nevertheless, almost all MSN have a low level of labeling within their sparse cytoplasm. Since the majority of immunoreaction product is deposited on intracellular membranes, the low level of labeling in the majority of striatal neurons might reflect their relatively limited cytoplasm and organelles. A cytoplasmic distribution is at odds with that reported in mouse hippocampus (Thompson et al., 2002) where no NR2D subunit immunoreactivity was found in perikarya. Although receptors might be expected to be associated with synaptic specializations, receptor subunits are trafficked through the endoplasmic reticulum where they may undergo a type of quality control (Fleck, 2006). It seems likely, therefore, that localization of protein to the endoplasmic reticulum reflects the distribution of the majority of these subunits within a cell. Subunit protein was not apparently enriched on synaptic membranes suggesting that few synapses on striatal cholinergic neurons express functional NRs incorporating NR2D subunits at any one time, although this may be related to a rapid turnover rate, with most subunits held in reserve.

Properties conferred by NR2D subunits

Striatal cholinergic interneurons are characterized by their tonic firing pattern which is interrupted during a conditioned movement (Graybiel et al., 1994). This distinctive firing is not simply related to their specific excitatory inputs (Lapper and Bolam, 1992; Zhou et al., 2002) since other striatal interneuron populations receive similar glutamatergic inputs (Bennett and Bolam, 1994; Rudkin and Sadikot, 1999; French et al., 2005). A unique population of glutamatergic receptors might provide a mechanism for specific regulation of cholinergic interneurons by these glutamatergic afferents. Compared with receptors with the NR2A subunit, those consisting of NR1, NR2B and NR2D subunits have high affinity for glutamate (Priestley et al., 1995; Dingledine et al., 1999) compatible with the relatively low levels of glutamatergic innervation mediating a profound effect on these neurons. The presence of NR2D subunits means that cholinergic neurons would experience significant calcium influx when they are at or near resting potential, perhaps compensating functionally for their lack of AMPA GluR2/3 receptors (Arruda Paes et al., 2004).

CONCLUSIONS

The NR2D subunit of NRs is strongly expressed by striatal cholinergic neurons. These interneurons very likely express NRs made up of NR1, NR2B and NR2D subunits

and are relatively insensitive to magnesium while responding to low levels of glutamate itself. Their different calcium regulation, combined with the ability to take up extracellular glutamate via vesicular glutamate transporter (Fremeau et al., 2002; Gras et al., 2002), might explain their relative resistance to degeneration in Huntington's disease (Chen et al., 1996; Hynd et al., 2004).

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