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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.

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 (Momiyama 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.

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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 Na_2HPO_4 (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