Differential sensitivity of medium- and large-sized striatal neurons to NMDA but not kainate receptor activation in the rat

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Abstract

Infrared videomicroscopy and differential interference contrast optics were used to identify medium- and large-sized neurons in striatal slices from young rats. Whole-cell patch-clamp recordings were obtained to compare membrane currents evoked by application of N-methyl-D-aspartate (NMDA) and kainate. Inward currents and current densities induced by NMDA were significantly smaller in large- than in medium-sized striatal neurons. The negative slope conductance for NMDA currents was greater in medium- than in large-sized neurons and more depolarization was required to remove the Mg2+ blockade. In contrast, currents induced by kainate were significantly greater in large-sized neurons whilst current densities were approximately equal in both cell types. Spontaneous excitatory postsynaptic currents occurred frequently in medium- than in large-sized neurons. A final set of experiments assessed a functional consequence of the differential sensitivity of medium- and large-sized neurons to NMDA. Cell swelling was used to examine changes in somatic area in both neuronal types after prolonged application of NMDA or kainate. NMDA produced a time-dependent increase in somatic area in medium-sized neurons whilst it produced only minimal changes in large interneurons. In contrast, application of kainate produced significant swelling in both medium- and large-sized cells. We hypothesize that reduced sensitivity to NMDA may be due to variations in receptor subunit composition and/or the relative density of receptors in the two cell types. These findings help define the conditions that put neurons at risk for excitotoxic damage in neurological disorders.

Introduction

The striatum is composed primarily of medium-sized spiny projection neurons (Jiang & North, 1991; Gerfen, 1992). These cells make up >95% of the total number of striatal neurons and they have been extensively studied. The other neuronal subtypes, medium- and large-sized interneurons, are less numerous and because of their scarcity they have not been extensively studied from a functional perspective. Whilst there are several subpopulations of interneurons, one subtype, the large cholinergic neuron, has been the subject of much interest because it is the source of acetylcholine in the striatum (Bolam et al., 1985; Di Chiara et al., 1994).

Until recently it has been difficult to study large cholinergic neurons electrophysiologically because they are scarce (Graveland & DiFiglia, 1985) and rarely sampled with standard in vivo or in vitro techniques (Wilson et al., 1990). The advent of infrared videomicroscopy and differential interference contrast optics (IR-DIC), allowing visual identification of different cell types during brain slice experiments (Dodt & Zieglgänsberger, 1994), makes recording from these neurons an easier task (Kawaguchi, 1993; Bennett & Wilson, 1998, 1999). Briefly, in vivo these neurons display slow spontaneous firing and low amplitude short latency excitatory postsynaptic potentials (EPSPs) to cortical and thalamic stimulation. In vivo and in vitro large cholinergic interneurons are more depolarized than medium-sized projection neurons and show time-dependent rectification (Wilson et al., 1990; Jiang & North, 1991; Kawaguchi, 1993). Neuronal inputs from the cerebral cortex appear to be considerably reduced compared to those on medium-sized neurons, whereas a prominent thalamic projection which is likely to be excitatory has been demonstrated (Lapper & Bolam, 1992). These large interneurons express multiple subtypes of glutamate receptors (Chen et al., 1996; Standaert et al., 1996). Excitatory postsynaptic currents (EPSCs) induced by local intrastriatal stimulation can be mediated by activation of both N-methyl-D-aspartate (NMDA) and non-NMDA receptors (Kawaguchi, 1992; Bennet & Wilson, 1998). Large cholinergic interneurons also express dopamine receptors (Bergson et al., 1995; Yan et al., 1997). Dopamine, via activation of D1 family receptors, produces an enhancement of excitability by suppression of K+ conductances and by modulation of the after-hyperpolarization (Aosaki et al., 1998; Bennet & Wilson, 1998).

One important property of large cholinergic interneurons is that, unlike medium-sized spiny striatal neurons, they are spared from
neurodegeneration in Huntington’s disease, a genetically based neurodegenerative disorder (Ferrante et al., 1985; Kowall et al., 1987). Large cholinergic interneurons also may be less vulnerable to ischemic challenge (Chesselet et al., 1990; Calabresi et al., 1997; Centonze et al., 2001). It has been hypothesized that glutamate receptor excitotoxicity is responsible for cell death of medium-sized neurons in Huntington’s disease because some of the neurochemical and neuropathological effects of this disorder are mimicked by application of glutamate receptor agonists (McGeer & McGeer, 1976; Beal et al., 1991). Potential explanations for sparing of large interneurons have implicated altered expression of normal or mutant huntingtin (Ferrante et al., 1997; Fusco et al., 1999) and/or hyporesponsiveness to glutamate-containing inputs compared to the medium-sized spiny neuron (DiFiglia, 1990). A previous study has provided some support for this explanation by demonstrating that large-sized striatal neurons are less responsive to glutamate receptor agonists than are medium-sized neurons (Calabresi et al., 1998).

Our laboratory has studied extensively electrophysiological and synaptic responses of medium-sized striatal neurons to activation of excitatory amino acid receptors (Cepeda et al., 1991, 1993, 1998a; Levine et al., 1996; Hurst et al., 2001). In addition, in our studies of medium-sized neurons we have used cell swelling, an index of early signs of excitotoxicity (Choi, 1992), to examine functional characteristics of excitatory amino acid receptors and their modulation by dopamine and activation of metabotropic glutamate receptors (Colwell & Levine, 1996; Colwell et al., 1996; Cepeda et al., 1998b). The present study was designed to directly compare the responsiveness of medium- and large-sized striatal neurons to activation of excitatory amino acid receptors using several approaches, whole-cell voltage-clamp analysis of current responses, synaptic activation and cell swelling in response to application of excitatory amino acid receptor agonists.

Methods

Animals

All procedures were carried out in accordance with the USPHS Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at UCLA. Sprague-Dawley rat pups (12–18 days old; n = 60) were used in these experiments. The choice of this age range was based on our experience with NMDA receptor function as determined by electrophysiological experiments or by cell-swelling experiments from visualized cells in striatal slices (Cepeda et al., 1995, 1998a; Colwell et al., 1996, 1998; Cepeda et al., 1998b). This age represents a compromise between the presence of excitatory amino acid-evoked responses, and the ability to visualize cells which becomes more difficult in tissue from older rats. It is approximately the same age range used in recent studies analysing electrophysiological responsiveness of large-sized striatal interneurons (Bennett & Wilson, 1998, 1999).

Preparation of slices

Procedures for tissue preparation, visualization, electrophysiology and measurement of cross-sectional areas in cell-swelling experiments have been described (Cepeda et al., 1995; Colwell & Levine, 1996; Colwell et al., 1996; Cepeda et al., 1998b). Briefly, rats were anaesthetized with halothane and then killed by decapitation. After dissection, brains were placed in cold oxygenated artificial cerebrospinal fluid (ACSF1) containing (in mM) NaCl, 130; NaHCO3, 26; KCl, 3; MgCl2, 5; NaH2PO4, 1.25; CaCl2, 1.0; glucose, 10 (pH 7.3–7.4). Transverse striatal sections were cut (350 μm) and placed in oxygenated (95%O2–5% CO2) ACSF2 (differing from ACSF1 as follows: CaCl2, 2 mM; MgCl2, 2 mM; lactate, 4 mM) at 25–27 °C for at least 1 h, and then transferred to a perfusion chamber attached to

Fig. 1. (A) An IR image of a patch pipette attached to a large-sized striatal neuron (white arrow). A medium-sized striatal neuron is next to the large-sized neuron (white double arrows). (B and D) IR images of a medium- (B) and a large-sized (D) neuron. The same cells are shown in C and E after biocytin processing. Scale bar in A also applies to B and D; in E also applies to C.
the fixed-stage of an upright microscope (Zeiss Axioskop, Thornwood, New York, USA) in which the slice was submerged in continuously flowing oxygenated ACSF2 (25 °C, 4 mL/min, lactate removed). Cells were visualized with a 40× water-immersion objective, illuminated with near IR light (790 nm, Ealing Optics, Holston, MA, USA) and the image detected with an IR-sensitive CCD camera. Digital images were stored for subsequent analysis when necessary. Cells were typically visualized from 30 to 100 μm below the surface of the slice. It was relatively easy to distinguish medium- and large-sized cells. The somatic area of medium-sized cells was ≈ 100 μm² whereas the somatic area of large cells was typically >150 μm² (Fig. 1).

**Whole-cell voltage-clamp**

Patch electrodes (3–6 MΩ) were filled with one of the following internal solutions, depending on the purpose of the experiment, (in mM): K-glucuronate, 140; HEPES, 10; MgCl2, 2; CaCl2, 0.1; ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid (EGTA), 1.1; and K2ATP 2 or Cs-methanesulphonate, 125; NaCl; KCl, 3; MgCl2, 1; MgATP, 5; EGTA, 9; HEPES, 8; GTP, 1; phosphocreatine, 10 and leupeptin, 0.1 (pH 7.2–7.3; osmolality 280–290 mOsm).

Unless otherwise noted, tetrodotoxin (1 μM) was added to the external solution to block Na⁺ currents after the whole-cell configuration was obtained. In experiments in which responses to bath application of excitatory amino acid receptor agonists during injection of ramp command voltages were examined, a CsF-based solution was used in order to block voltage-gated Ca²⁺ currents. It contained (in mM): CsF, 125; NaCl; 4; MgCl2, 1; EGTA, 9; HEPES, 8; and K₂ATP, 5. To block K⁺ currents, tetraethylammonium (20 mM) was in the external solution.

Axopatch 200A or 1D amplifiers were used for voltage-clamp recordings. A 3-M KCl-agar bridge was inserted between the extracellular solution and the Ag–AgCl indifferent electrode. Tight seals (2–10 GΩ) from visualized large- and medium-sized cells were obtained by applying negative pressure. The membrane was disrupted with additional suction and the whole-cell configuration was obtained. The access resistances ranged from 8 to 15 MΩ. Series resistances were compensated 60–85%.

Cell capacitance measurements as well as somatic area were used to distinguish between large- and medium-sized neurons. Once the whole-cell configuration was obtained, the capacitance of the cell was calculated by using a 10-mV hyperpolarizing pulse (40 ms duration). In experiments in which excitatory amino acid receptor agonists were bath-applied, the magnitude of the current was examined in response to a ramp. The ramp consisted of a voltage command from −70 to −90 mV over 0.4 s, followed by a ramp to +40 mV over 6.4 s to ensure that unblocked or poorly blocked Ca²⁺ channels were inactivated. At +40 mV, the holding potential was followed by a ramp command to −90 mV over 0.9 s. Current responses in the absence of excitatory amino acid receptor agonists were subtracted from responses in the presence of bath-applied NMDA or kainate to isolate specific agonist-induced currents. Isolated currents were plotted against voltage to determine current–voltage relationships (Figs 2 and 3). Currents were converted to current density by dividing by cell capacitance to normalize responses between the different cell sizes to better permit comparison of data across experimental conditions (Alzheimer et al., 1993). Both upward (−90 to +40 mV) and downward (+40 to −90 mV) ramps were used to generate current–voltage plots. Currents obtained from the upward and downward ramps were similar and the downward ramp was used when data were quantified (Burgard & Hablitz, 1994). NMDA (100 μM) and kainate (100 μM) were bath-applied for 3 min prior to applying the ramp protocol. Each application of either NMDA or kainate was followed by a 5–10 min washout. We used kainate instead of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) because large interneurons have been shown to express kainate but not AMPA receptor subunits (Chen et al., 1996), although a more recent single-cell analysis indicated that AMPA receptor subunit proteins are expressed (Richardson et al., 2000).

Whole-cell voltage-clamp data, especially quantitative estimates of currents from neurons in slices, should be interpreted with caution because the currents measured at the soma are undoubtedly distorted as a result of the nonisopotentiality over the neuronal surface due to space-clamp limitations (Armstrong & Gilly, 1992). To partially control for this problem we also examined responses in acutely dissociated neurons.

**Electrical stimulation**

A glass pipette filled with the external solution was placed = 200 μm from the recording pipette. Single pulses (200 μsec duration) were delivered every 10–20 s. The stimulus intensity was adjusted to 2× the threshold to evoke synaptic responses. Four to six pulses were applied at different holding potentials and averaged. Excitatory postsynaptic currents (EPSCs) mediated by activation of glutamate receptors were isolated by adding bicuculline methiodide (10 μM), a GABA_A receptor blocker, to the bath solution.

**Acute neuron dissociation**

Some slices also were used for acute dissociation of neurons. Slices were incubated for 1–6 h at room temperature in NaHCO₃-buffered saline bubbled with 95% O₂/5% CO₂ (in mM except where otherwise noted): NaCl, 126; KCl, 2.5; MgCl₂, 2; CaCl₂; NaHCO₃, 26; Na₂HPO₄, 1; pyruvic acid, 1; glutathione 5 μM; N⁵-nitro-l-arginine, 1; kynurenic acid, 1; glucose, 10; HEPES, 15; pH 7.4 with NaOH, 300–305 mOsm/L. After 1 hr incubation, a slice was placed in low-Ca²⁺-isethionate solution (in mM): NaCl, 126; KCl, 2.5; MgCl₂, 2; CaCl₂; NaHCO₃, 26; Na₂HPO₄, 1; glucose, 10; HEPES, 15; pH 7.4, 300–305 mOsm/L, and the dorsal striatum was dissected and placed in an oxygenated cell-stir chamber (Wheaton, Inc., Millville, NJ USA) containing papain (Calbiochem, La Jolla, CA USA; papain, 1–2 mg/mL) in HEPES-buffered Hanks’ balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, MO, USA) at 35 °C. After 20–40 min of enzyme digestion, tissue was rinsed three times with the low-Ca²⁺-isethionate solution and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was plated into a 35-mm NUNCCLON dish containing HEPES-buffered HBSS saline on the microscope stage.

Whole-cell recordings from dissociated neurons used standard techniques (Hamill et al., 1981; Bargas et al., 1994). The internal solution consisted of (in mM): N-methyl-d-glucamine, 180; HEPES, 40; MgCl₂, 2; EGTA; 10; phosphocreatine, 12; Na₂ATP, 2; Na₃GTP, 0.2; leupeptin, 0.1; pH 7.2–7.3. In the presence of bath-applied NMDA or kainate to isolate specific agonist-induced currents. The external solution consisted of (in mM): NaCl, 135; CsCl, 20; BaCl₂, 5; glucone, 10; HEPES, 10; tetrodotoxin, 0.001; glycine, 0.02; pH 7.3 with NaOH, 300–305 mOsm/L.

Recordings from acutely dissociated neurons were obtained with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Electrode resistance was 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. Recordings were made only from medium- or large-sized cells that had short (< 75 μm) proximal dendrites. NMDA (1, 10, 50, 100, 200 or 1000 μM, 3 s duration every 20 s) was applied with a gravity-fed ‘two-pipe’ system. The array of application capillaries (= 150 μm i.d.) was positioned a few hundred μm from the...
cell under study. Solution changes were effected by changing the position of the array with a DC drive system, controlled by a SF-77B perfusion system (Warner Instruments Co., Hamden, CT, USA) synchronized by pClamp. Solutions could be delivered within 5±10 ms with this system.

Cell identification
In most experiments, electrodes were filled with 0.2% Biocytin (Sigma, St Louis, MO, USA) in the internal solution. After the experiment, the slice was fixed in 4% paraformaldehyde overnight, then processed according to published protocols (Horikawa & Armstrong, 1988).

Cell swelling
Preparation of brain slices for these experiments were the same as those described for electrophysiology except tetrodotoxin and tetraethylammonium were omitted from the bath. After equilibration for 10 min, a baseline image of the cells was obtained and stored. The slice was then exposed to different solutions depending upon the purpose of the specific experiment. Images were obtained and stored at 5-min intervals for the duration of each experiment (15 min exposure to experimental treatment). In order to quantify changes in response to excitatory amino acid receptor agonists, cross-sectional somatic area was measured at each 5-min time point, before and during experimental treatments. Each measurement was made two or three times on each image and the average value recorded for each cell at each time point. For each experimental group, data were obtained from several animals. Typically, 3–7 medium-sized cells were visualized within each slice whilst large cells were sparse so that usually only one cell could be analysed in each slice. In slices containing large-sized neurons, neighbouring medium-sized cells were also measured. Cross-sectional areas at each time point were converted to percentage change with respect to the baseline area for each cell. For statistical analyses, data were pooled for all cells in experimental and control groups. Concentrations of NMDA (50 and 100 μM) or kainate (100 μM) were based on our previously published studies (Colwell & Levine, 1996; Cepeda et al., 1998b). Drugs were applied in the bath and freshly prepared each day.

Statistics
Values in the tables, figures and text are presented as means ± SEM. Differences among group means were assessed with appropriate t-tests or analyses of variance (ANOVAS). Welch’s approximation to the t-test for unequal variances was used when group variances were not homogeneous (Welch, 1947). For post hoc evaluations using ANOVAs, the Bonferroni t-test was used because this test is one of the more conservative approaches using multiple comparisons. In the text, only P-values and the type of test used are reported.
Results

Neuron identification

Both medium- and large-sized neurons could be visualized easily in the slices (Fig. 1). Cross-sectional areas were significantly smaller for the medium-sized neurons (Fig. 1, compare B vs. D and C vs. E) [103 ± 5 μm² (n = 15) vs. 327 ± 13 μm² (n = 21)] for medium- and large-sized neurons, respectively; t-test, P < 0.001]. Dendritic processes were examined after filling the neuron with biocytin. Medium-sized neurons typically displayed dendritic fields with spine-like varicosities (Fig. 1C), although spines were not abundant due to the young age of the rats (Hattori & McGee, 1973; Tepper et al., 1998). Large-sized neurons exhibited dendritic branches with or without scarce varicosities (Fig. 1E). Although several types of large neurons have been described in striatum, the present study targeted the very large, putative cholinergic, interneurons (Kawaguchi, 1993). These cells more probably correspond to the so-called ‘Type 1’ large neurons (Bolam et al., 1984). Because we did not identify the large cells immunohistochemically, we use the term large-sized neurons rather than cholinergic interneurons to describe these cells.

NMDA- and kainate-induced currents during ramp voltage commands

Whole-cell voltage-clamp recordings were obtained from 30 neurons (15 medium- and 15 large-sized) exposed to bath application of NMDA (100 μM). For both large- and medium-sized neurons ramp voltage commands produced a typical current–voltage function to NMDA application (Fig. 2A). Lower amplitude currents were produced in large- compared to medium-sized neurons (Fig. 2A and B). These differences were statistically significant from −40 to −25 mV (ANOVA, post hoc t-tests, P < 0.05; Fig. 2B). Capacitance measurements for medium- (48.3 ± 3.1 pF) and large-sized (83.8 ± 5.7 pF) neurons reflected the difference in cross-sectional area and were used to calculate current densities. It is important to take into account cell size or capacitance when evaluating ionic conductances in neurons of different sizes because the absolute magnitude of the current can be a function of neuronal size and not necessarily the density of channels (Alzheimer et al., 1993). The differences between mean current densities for large- and medium-sized neurons were proportionately much greater than the differences between current amplitudes (Fig. 2C). These differences were statistically significant from −60 to −10 mV (ANOVA, post hoc t-tests, P < 0.05). In addition, the voltages at which peak currents were obtained were significantly shifted to more depolarized potentials in the large-sized neurons (−27.0 ± 1.0 mV vs. −30.4 ± 1.2 mV for large- and medium-sized cells, respectively; t-test, P < 0.025; Fig. 2A–C).

Because the slope of the current–voltage plots of the NMDA-induced currents in large- and medium-sized neurons appeared different, we examined more closely the voltage-dependence of NMDA receptor activation. The current–voltage relationship was converted to a conductance–voltage relationship (G–V) according to the formula $G = I/(V_m - V_{rev})$, where $V_m$ is the membrane potential and $V_{rev}$ is the potential where the current was observed to reverse polarity. The midpoint potential ($V_{0.5}$) and slope factor ($b$) of the G–V relationship were calculated by fitting the data obtained from individual neurons by $G(G_{max}) = 1/(1 + e^{(V_{0.5} - V_{mem})/b})$, where $V_{0.5}$ is the potential at which $G = 0.5$ and $b$ is the number of mV required to cause an e-fold change in conductance. According to this function, the smaller the value of $b$, the fewer mV are needed to cause an e-fold change in conductance. In other words, the smaller the value of $b$, the steeper the slope of the G–V relationship. Mean conductances ($G$) were significantly greater for medium- than for large-sized neurons from −35 to −20 mV (Fig. 2D) (ANOVA, post hoc t-tests $P < 0.05$). The slope also was significantly greater in medium-than in large-sized neurons (13.1 ± 0.8 vs. 11.0 ± 0.7, respectively; t-test, $P < 0.05$). The midpoint of the $G$–$V$ relationship was $±10$ mV more negative in medium- than in large-sized neurons (−38.3 ± 1.85 vs. −30.5 ± 2.1 mV, respectively, t-test, $P < 0.05$) indicating that NMDA receptors of large-sized neurons required more depolarization to remove the Mg²⁺ block.

Kainate (100 μM)-induced currents were measured in 10 large- and 10 medium-sized cells. For both large- and medium-sized neurons, ramp voltage commands produced a typical linear current–voltage function to kainate application (Fig. 3A and B). In contrast to NMDA application, higher amplitude currents were produced in large- vs. medium-sized neurons. These differences were statistically significant from −90 to −30 mV (ANOVA, post hoc t-tests, $P < 0.05$; Fig. 3B). Capacitance measurements for medium- (53.4 ± 4.3 pF) and large-sized (107.3 ± 6.3 pF) neurons were used to calculate current densities. The differences between mean current densities for large- and medium-sized neurons were proportionately smaller and were not statistically significant (Fig. 3C).

Acutely dissociated neurons

Concentration–response relationships to activation of NMDA receptors were examined in more detail in acutely dissociated large- and medium-sized neurons using a rapid perfusion system so that neurons could be tested with a series of concentrations of NMDA. Data were obtained from 12 medium- and 12 large-sized neurons (Fig. 4A). All neurons were held at −40 mV in Mg²⁺-free ACSF to remove the voltage-dependence of the response. Neurons were exposed to a series of ascending concentrations of NMDA (1, 10, 50, 100, 200 and 1000 μM, 3 s duration). Medium-sized neurons displayed a rapid peak current followed by a steady-state response (Fig. 4B). Large-sized neurons displayed a smaller peak response than did medium-sized neurons, and this was also followed by a steady-state response (Fig. 4C). Concentration–current response relationships were sigmoidal and medium-sized neurons displayed greater peak currents at 50, 100, 200 and 1000 μM concentrations (Fig. 4D). However, because not all neurons were tested at each concentration the difference was only statistically significant at the 100-μM concentration (t-test, $P < 0.05$). The respective EC₅₀ were 49.5 ± 9.8 and 38.2 ± 22.4 μM for medium- vs. large-sized neurons. Current densities for peak responses were also computed by dividing by cell capacitances (Fig. 4E, inset). There were much greater increases in concentration–current density responses for medium- than for large-sized neurons at 50, 100, 200 and 1000 μM concentrations (Fig. 4E). The differences were statistically significant for each of these concentrations (t-tests, $P < 0.05$ to $P < 0.01$). The respective EC₅₀ were 33.3 ± 14.6 and 14.7 ± 14.1 μM for medium- and large-sized neurons, respectively. Mean steady-state currents at the 100-μM concentration were greater for medium- than for large-sized neurons (−329 ± 60 vs. −196 ± 26 pA, respectively), but the difference was not statistically significant. In contrast, steady-state current densities at the 100-μM concentration were significantly greater for medium- vs. large-sized neurons (−54 ± 10 vs. −12 ± 2 pA/pF, respectively; t-test, $P < 0.001$).

Spontaneous and evoked excitatory postsynaptic currents

Spontaneous EPSCs were examined in large- and medium-sized neurons in the presence of bicuculline (10 μM), a GABA_A receptor blocker. Spontaneous currents were sampled from 30 s up to 15 min in individual neurons at a holding potential of −60 mV. These
currents (> 5 pA in amplitude) occurred frequently in medium-sized neurons (Fig. 5B, left trace). In marked contrast, few spontaneous events were present in large-sized neurons (Fig. 5B, right trace). The difference in mean frequency was statistically significant (1.64 ± 0.42 vs. 0.05 ± 0.03 events/s in medium- and large-sized neurons, respectively; t-test, \( P < 0.05 \)). Average amplitudes of events were similar in medium- and large-sized neurons (11.5 ± 0.89 and 9.2 ± 0.58 pA, respectively; difference not statistically significant). The decrease in frequency of occurrence of spontaneous currents probably reflects less afferent input to large- compared to the medium-sized neurons (Bolam et al., 1984).

Electrical stimulation in close proximity to the cell was used to evoke EPSCs in medium- and large-sized neurons. The mean currents required to evoke EPSCs at 2\( \times \) threshold in large- and medium-sized neurons were similar (29.5 ± 9.0 and 20.0 ± 1.43 nA, respectively; difference not statistically significant). EPSCs were evoked at three holding potentials: −60, −20 and +30 mV. To analyse the NMDA receptor-mediated component 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 \( \mu \)M) to block non-NMDA receptors was added and the membrane was held at −20 mV. Peak currents at −60 mV holding potentials (in the absence of CNQX and mediated primarily by activation of non-NMDA receptors) did not differ significantly between medium- and large-sized neurons (−169 ± 42 and −110 ± 21 pA, respectively). There was a statistically significant difference between cell capacitances for medium- and large-sized neurons (44 ± 7.1 vs. 94 ± 13.9 pF, respectively; t-test, \( P < 0.005 \)). To evaluate peak current densities, peak currents were divided by cell capacitance. There was a statistically significant decrease in current density in the large- compared to the medium-sized neurons (−1.4 ± 0.3 vs. −3.9 ± 0.6 nA/pF, respectively; t-test, \( P < 0.001 \)). EPSCs mediated by activation of NMDA receptors (peak currents measured at −20 mV holding potential in the presence of CNQX) in medium-sized neurons were not significantly greater in amplitude than those in large-sized neurons (−30.4 ± 7.8 vs. −16.4 ± 3.3 nA, 1582 C. Cepeda et al. © 2001 Federation of European Neuroscience Societies, European Journal of Neuroscience, 14, 1577–1589

**Fig. 3.** Responses of medium- and large-sized neurons to kainate (100 \( \mu \)M) application. (A) Current–voltage plots for typical medium- and large-sized neurons. A ramp command was used to change voltage from −90 to +40 mV and back to −90 mV. The plotted data were obtained from the downward ramp. Control current–voltage plots were subtracted from plots in the presence of kainate. (B) Current–voltage plots (mean ± SEM) for medium- and large-sized neurons. (C) Current density–voltage plots for medium- and large-sized neurons. In B asterisks indicate groups differences were statistically significant (\( P < 0.05 \)). See Results for further details.
FIG. 4. (A) Medium- and large-sized striatal neurons after acute dissociation and with the patch electrodes attached. Calibration in right panel refers to both panels. (B and C) Current traces evoked by 3-s application of NMDA at 1, 10, 50, 100, 200 and 1000 μM concentrations in (B) medium- and (C) large-sized neurons. (D) Concentration–peak current curves for medium- and large-sized neurons. (E) Concentration–peak current density curves for medium- and large-sized neurons. Inset shows mean cell capacitances (± SEM). In D and E, asterisks indicate groups differences were statistically significant (P < 0.05 to P < 0.01). See Results for further details. Scale bars in C also apply to B.
respectively). However, mean current densities for NMDA receptor-mediated responses were statistically significantly greater in medium- vs. large-sized neurons (−0.64 ± 0.10 vs. −0.20 ± 0.04 pA/pF, respectively; t-test, P < 0.001). In selected experiments (n = 3) we were able to record from pairs of large- and medium-sized neurons, using the same stimulation placement and parameters (Fig. 5A). Similar to the grouped data described above, spontaneous EPSCs were more frequent and evoked EPSCs recorded at each holding potential were of greater amplitude in the medium- than in the large-sized neurons (Fig. 5B and C).

**FIG. 5.** (A) Top left panel shows IR images of a pair of striatal cells; one is a medium-sized (double arrow) and the other is a large-sized neuron (single arrow). Top right panel shows cells after being filled with biocytin. Single arrow points to the large neuron and the double arrow to the medium-sized neuron. (B) Patch-clamp recordings were obtained from both cells. Spontaneous EPSCs were recorded in the presence of bicuculline (10 μM) at a holding potential of −60 mV. Middle traces are slow-speed recordings illustrating the marked absence of spontaneous EPSCs in the large-sized neuron (right) compared to the medium-sized cell (left). (C) A stimulating electrode was placed 200 μm from the cells, thus approximately equidistant. Bottom traces are faster speed recordings showing evoked EPSCs at three holding potentials: −60, −20 and +30 mV. The stimulation parameters were identical for both cells. In all cases the evoked EPSCs were of greater amplitudes in the medium-sized neuron. Downward arrows indicate time of occurrence of stimulation pulse.
Cell swelling

Another approach to examining excitatory amino acid receptor function in large- and medium-sized striatal neurons is to expose slices to agonists and assess cell swelling. Cell swelling, induced by activation of excitatory amino acid receptors, is presumably the first step in a cascade that may ultimately lead to cell death (Choi, 1992). Previously, we showed that bath application of NMDA or kainate produced dose- and time-dependent swelling of medium-sized striatal cells (Colwell & Levine, 1996; Cepeda et al., 1998b). In the present experiments we examined the ability of large-sized striatal neurons to swell in the presence of NMDA or kainate. Modal cross-sectional areas for large cells were between 250 and 350 \( \mu \text{m}^2 \) (Fig. 6B, inset). NMDA (50 or 100 \( \mu \text{M} \)) induced almost no change in cross-sectional area in large-sized striatal neurons (Fig. 6A, top panels). Average percentage increases in area were <5% over the 15-min assessment period (Fig. 6B). When a group of medium-sized neurons (n = 15) was exposed to the same concentration of NMDA, statistically significant cell swelling occurred. After 5 min exposure to NMDA there was a 19 ± 3% increase in somatic cross-sectional area (ANOVA, post hoc t-test, \( P < 0.001 \)). By 15 min the somatic cross-sectional area increased by 43 ± 6% (ANOVA, post hoc t-test, \( P < 0.001 \)). Because it is possible that large-sized cells may be unable to increase somatic area, we tested the response to kainate in another group of large-sized neurons. Kainate (100 \( \mu \text{M} \)) produced a statistically significant increase (40%) in large-sized cell cross-sectional area 5, 10 and 15 min after application (ANOVA, \( P < 0.003 \)) (Fig. 6A, bottom panels, and 6B). Thus, the lack of response to NMDA was not due to an inability of large-sized cells to swell in the presence of excitatory amino acid receptor agonists. In medium-sized neurons we have shown previously that 100 \( \mu \text{M} \) kainate produces about a 30% increase in cross-sectional area (Colwell et al., 1998).

We have shown previously that dopamine via activation of D1-family receptors increases cell swelling induced by excitatory amino acid receptor agonists in medium-sized striatal neurons (Cepeda et al., 1998b). Thus, we examined whether NMDA in combination with dopamine could produce cell swelling in large-sized neurons (n = 5 cells). In the presence of dopamine (50 \( \mu \text{M} \)), cell swelling was not significantly increased (Fig. 6B).

Discussion

The present experiments demonstrate, using a number of electrophysiological approaches, that large-sized striatal neurons were less responsive to NMDA receptor activation than were medium-sized neurons. First, whole-cell currents and to an even greater extent current densities, induced by exogenous application of NMDA, were decreased in large- compared to medium-sized neurons. Comparable findings occurred in acutely dissociated neurons. Peak and steady-state current densities to NMDA application were reduced in large- compared to medium-sized neurons and this effect was consistent across a series of concentrations. There was also a shift in the voltage-dependence toward less negative values in large- compared to medium-sized neurons and a less steep negative slope conductance. In contrast, kainate-induced currents were greater in large- than in medium-sized neurons whilst kainate-induced current densities were similar in both cell types. In addition to decreased responsiveness to exogenous application of NMDA, large-sized neurons received less excitatory synaptic input than medium-sized neurons. Spontaneous EPSCs were markedly reduced in frequency and evoked EPSCs mediated by activation of NMDA or non-NMDA receptors were reduced in amplitude. Finally, NMDA-induced cell swelling, a first step in an excitotoxic cascade, does not occur or is minimal in large-sized neurons. This was true even in the presence of dopamine, a treatment that enhances NMDA-induced cell swelling in medium-sized neurons (Cepeda et al., 1998b). In contrast, kainate-induced swelling was robust in large-sized neurons.

It is clear from the present findings as well as previous analyses (Calabresi et al., 1998) that large-sized cholinergic neurons display reduced responsiveness to activation of NMDA receptors compared to medium-sized neurons. There are a number of mechanisms that could underlie such reduced responsiveness. These include decreased density of receptors and/or altered NMDA receptor subunit composition. The results of the present study clearly demonstrate that current density, which is a measure of current per unit of membrane clamped, is significantly reduced in large-sized interneurons compared to the medium-sized cells, suggesting lower density of receptors. Whilst these values will be affected by differential space-clamp limitations to a certain extent, the magnitude of the difference cannot be accounted for by space-clamp errors.

Of at least equal importance is the difference in NMDA receptor subunit composition between large- and medium-sized neurons (Landwehrmeyer et al., 1995). Differences in subunit composition will affect the agonist affinity, Mg\(^{2+}\) sensitivity, voltage-dependence and the kinetics of the responses. For example, the lack of a large rapid peak response to NMDA application in dissociated large-sized neurons is probably due to differences in subunit composition (Huganir & Greengard, 1990). Large-sized cholinergic interneurons express lower levels of NMDA-R1 and NMDA-R2B subunit mRNA than do enkephalin-positive medium-sized neurons, and preferentially express the NMDA-R1 splice variant forms lacking one alternatively spliced carboxy-terminal region (Landwehrmeyer et al., 1995), which would change the sensitivity of protein phosphorylation (Tingley et al., 1993). The large-sized cholinergic interneurons also do not express NMDA-R2A subunits (Standaert et al., 1996). This finding is relevant because NMDA-R2 subunits impart different pharmacological profiles to NMDA receptors (Monaghan & Larsen, 1997), and residues on the NMDA-R2A subunit control glutamate potency (Anson et al., 1998). Finally, the NMDA-R2D subunit is selectively expressed in striatal interneurons and absent in medium-sized projection neurons (Standaert et al., 1996, 1999). Channels expressing this subunit are unique in that they have a very long offset decay and an altered Mg\(^{2+}\) blockade (Monyer et al., 1994) which may explain, in part, the reduced negative slope conductance of the NMDA current. Furthermore, cells expressing NMDA-R2D subunits may have lower affinity for agonists (Buller et al., 1994) which could partially explain sparing of the large neurons after quinolinic acid lesions. Recent studies using single-cell PCR have corroborated and extended the initial analyses of NMDA receptor subunit composition in large-sized cholinergic interneurons (Richardson et al., 2000). Interestingly, the NMDA-R2A subunit was detected when the sensitivity of the single cell assay was increased, suggesting low levels of expression.

There are also fewer excitatory synaptic inputs to large- than to medium-sized neurons. The present data confirm and extend the initial analyses demonstrating that these cells receive less excitatory input than do medium-sized spiny neurons (Wilson et al., 1990; Bennet & Wilson, 1999). We showed both reduced frequency of spontaneous EPSCs and reduced amplitudes of evoked EPSCs. Morphological evidence indicates fewer cortical excitatory synaptic inputs on large-sized interneurons than on other striatal cell types, although a thalamostriatal projection which is probably excitatory has...
been demonstrated (Lapper & Bolam, 1992). Based on electron microscopic studies, synaptic input to the perikarya and proximal dendrites of large cholinergic interneurons is very sparse and consists predominantly of boutons forming putative inhibitory symmetrical...
synaptic contacts. Asymmetric excitatory synaptic contacts are infrequent near the soma, although their number increases in distal dendrites (Bolam et al., 1984).

A previous study described a generalized decrease in responsiveness of large cholinergic interneurons to NMDA, AMPA and kainate (Calabresi et al., 1998). Paradoxically, a large NMDA receptor-mediated EPSP was expressed in the cholinergic interneurons in the presence of Mg²⁺ in the external solution, even at relatively hyperpolarized membrane potentials (−75 mV). The electrophysiological data in the present study do not support the indiscriminate hyposensitivity of large-sized neurons, at least for kainate receptor activation. Neither do they support the presence of a large NMDA receptor-mediated synaptic component at hyperpolarized potentials. In fact, our results indicate that more depolarization is required to remove the Mg²⁺ blockade in large interneurons and the NMDA receptor-mediated synaptic current is reduced. Calabresi et al. (1998) only examined peak voltages or currents and did not include measurements of current density; this could significantly alter the interpretation of the outcomes because large- and medium-sized neurons have markedly different surface areas. The present study has examined, in more detail and more extensively, current density and current–voltage relationships as well as directly comparing synaptic responses using voltage-clamp techniques in which changes in other variables would contribute less to the overall currents than in the synaptic responses in the previously reported current-clamp study. Calabresi et al. (1998) also did not initially block GABA_A receptors when examining the excitatory amino acid receptor-mediated EPSPs, and consequently the electrical stimulation produced a mixed depolarizing potential consisting of multiple components due to activation of ligand- and voltage-gated channels. As pointed out above, other studies have shown that, relative to medium-sized spiny neurons, the EPSP in large interneurons is smaller (Wilson et al., 1990) and, although it is mediated by activation of both NMDA and non-NMDA receptors, the NMDA component is negligible at hyperpolarized potentials (Kawaguchi, 1992). Although differences in maturation of large- vs. medium-sized neurons may have contributed to the disparity between the present findings and those of Calabresi et al. (1998), we believe this is unlikely because our findings on large-sized neurons are in general agreement for NMDA receptor function but contrast on kainate receptor function. We have shown previously that non-NMDA receptor function actually develops earlier in the striatum than do NMDA receptors (Colwell et al., 1998; Nansen et al., 2000; Hurst et al., 2001). Thus, at the ages used in the present study, kainate receptor function would be at least as mature as or more mature than NMDA receptor function.

Some of the unique properties of large-sized neurons are relevant for understanding their resistance to excitotoxic challenge, ischemia and their preservation in Huntington’s disease. For example, c-fos induction after NMDA receptor activation is absent in large interneurons but not in medium-sized neurons, suggesting less coupling of NMDA receptor function to gene expression in the large cells (Aronin et al., 1991). Large interneurons express kainate receptor subunit proteins (Chen et al., 1996) and the present results indicate that they respond to kainate application. It was originally reported that large interneurons do not express AMPA receptor subunits (Chen et al., 1996) although they respond to AMPA application (Calabresi et al., 1998). More recently, single-cell PCR analyses have demonstrated the presence of AMPA receptor subunit proteins (Richardson et al., 2000). Unfortunately, little is known about the subtypes of glutamate receptors activated by the thalamostriatal pathway.

Differential effects of anoxia–hypoglycemia occur between medium- and large-sized neurons in the striatum (Calabresi et al., 1997). Cholinergic interneurons hyperpolarize in response to anoxia–hypoglycemia whilst medium-sized neurons depolarize. Thus, they may be protected from the deleterious effects of depolarization induced by ischemic challenge. Whilst it was first hypothesized that activation of a K⁺ current that is insensitive to tolbutamide mediated the hyperpolarization (Calabresi et al., 1997), subsequent studies have demonstrated an ATP-sensitive K⁺ channel (Lee et al., 1997, 1998) that may provide a protective role in anoxia–hypoglycemia.

There are a number of reasons why large-sized cholinergic neurons may be spared in Huntington’s disease. First, there may be differences in expression of normal or mutant huntingtin in large-sized cholinergic interneurons (Ferrante et al., 1997). Selective vulnerability of medium-sized spiny striatal neurons observed in Huntington’s disease has been associated with higher levels of huntingtin expression, whereas the relative resistance of large- and medium-sized aspy neurons has been associated with low levels of huntingtin expression (Ferrante et al., 1997; Kosinski et al., 1997). However, a more recent study using a different antibody to identify huntingtin expression indicates that large cholinergic interneurons have higher levels of huntingtin than medium-sized neurons and suggests that it is the local environment that ultimately decides whether a neuron will be vulnerable to degeneration in Huntington’s disease (Fusco et al., 1999).

Early animal models of Huntington’s disease produced excitotoxic lesions with kainate to duplicate human striatal cell loss (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976). However, soon thereafter it was found that the excitotoxic model using quinolinic acid, which has more selectivity for NMDA receptors, better replicated the human findings (Schwarz et al., 1983; Beal et al., 1986; Ferrante et al., 1993; Roberts et al., 1993). If it is true that cholinergic interneurons are less sensitive to exogenous application of NMDA but not kainate as demonstrated by the present electrophysiological and cell swelling findings, how can we explain the sparing of these cells after kainate application? One possibility is that excitotoxic lesions in the striatum require two conditions, activation of NMDA receptors and a significant excitatory input (McGeer et al., 1978). The paucity of excitatory inputs impinging upon cholinergic interneurons may explain their relative resistance to kainate, whereas their resistance to NMDA could be explained by the combination of reduced receptor expression and different receptor subunit assembly, as well as paucity of excitatory inputs (Ferrante et al., 1987, 1993).

Fig. 6. (A) IR images. Top left and right panels show NMDA application (100 μM, 15 min duration) failed to produce cell swelling in the large neurons (white arrows). In the same slice, the medium-sized cells displayed significant cell swelling (white arrowheads). Bottom left and right panels show that, in contrast to the lack of swelling after NMDA, large neurons showed significant increases in somatic area after kainate (100 μM, 15 min duration) (white arrows). (B) Bar graphs showing lack of cell swelling in large-sized striatal neurons at 5, 10 and 15 min during application of NMDA at either 50- or 100-μM concentrations. In the presence of dopamine, there was a slight increase in cell swelling to 100 μM NMDA. In contrast, kainate (100 μM, 15 min duration) produced significant cell swelling after 5-, 10- and 15-min applications in large neurons. Inset shows a histogram of cross-sectional areas of large neurons. Asterisks in B indicate groups differences were statistically significant (P < 0.003). See Results for further details.
How are these results relevant for the understanding of Huntington’s disease or other neurodegenerative disorders? We believe that the present results emphasize that multiple conditions must be met before neurons are at risk for excitotoxic degeneration. Large-sized cholinergic interneurons do not meet these conditions and thus are spared both in excitotoxic models of Huntington’s disease and in the disease itself. In contrast, medium-sized spiny neurons meet both conditions and are at risk in both the models and the disorder.

In conclusion, the present study demonstrates that large interneurons in the striatum display unique responsiveness to NMDA receptor activation, differing from medium-sized neurons. Inward currents are significantly smaller and a stronger membrane depolarization is required to relieve the Mg$^{2+}$ blockade. Cell swelling to NMDA, a first step in an excitotoxic cascade, is practically nonexistent. In contrast, large-sized neurons respond to kainate receptor activation in a manner similar to medium-sized neurons. We also found that the distribution of excitatory inputs onto large interneurons is relatively sparse compared to medium-sized neurons. Thus, the reduced responsiveness coupled with the altered NMDA receptor composition or reduced receptor density may help explain the relative resistance of large interneurons to neurodegeneration in neurological diseases. Taken together the present findings help define the conditions that put neurons at risk for damage in neurological disorders and they will aid in the design of therapies that can reduce or prevent neurodegeneration.

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Abbreviations
ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethylene glycol-bis(β-aminoethoxy ether)-N,N,N′,N′-tetraacetic acid; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; HBSS, Hanks’ balanced salt solution; IR-DIC, infrared-differential interference contrast; NMDA, N-methyl-D-aspartate.

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