Long-Lasting NMDA Receptor-Mediated EPSCs in Mouse Striatal Medium Spiny Neurons

Stephen M. Logan,1,* John G. Partridge,1,* Jose A. Matta,2 Andres Buonanno,3 and Stefano Vicini1

1Departments of Physiology and Biophysics and 2Pharmacology, Georgetown University School of Medicine, Washington, DC; and 3Section on Molecular Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

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Logan SM, Partridge JG, Matta JA, Buonanno A, Vicini S. Long-lasting NMDA receptor-mediated EPSCs in mouse striatal medium spiny neurons. J Neurophysiol 98: 2693–2704, 2007. First published September 5, 2007; doi:10.1152/jn.00462.2007. Excitatory postsynaptic currents (EPSCs) from dorsolateral medium spiny neurons (MSNs) were recorded in cortico-striatal slice preparations from postnatal day 6–8 (P6-8) and >P12 wild-type mice and mice that were lacking either the NR2A or the NR2C subunit of the N-methyl-D-aspartate (NMDA) receptor. EPSCs were elicited by stimulation of the excitatory afferents and the NMDA and non-NMDA receptor-mediated components were pharmacologically isolated. The ratio of these components decreased with development and was significantly reduced only between age-matched +/+ and NR2A −/− neurons. In many MSNs, the NMDA-EPSC decay was characterized by the presence of a slow exponential component with a time constant lasting >1 s regardless of genotype or age. In the NR2A −/−, no developmental increase in the decay time (Tw) of the NMDA-EPSCs was observed although it was almost twofold longer than in +/+ MSNs. NR1/NR2B antagonists were ineffective in reducing the slow NMDA-EPSCs at all ages. Input-output studies revealed differences in stimulation threshold sensitivity of MSNs based on stimulus location. High-threshold responders were preferentially identified with stimulation from intracortical locations that produced considerably faster NMDA-EPSCs, whereas low-threshold responders were mainly elicited with stimulation more proximal to the striatum and exhibited slower NMDA-EPSCs. A low-affinity competitive antagonist of NMDA receptors failed to alter the decay of NMDA-EPSCs elicited from either location, suggesting that glutamate spillover is not responsible for the long-lasting NMDA-EPSCs. Our data are consistent with the expression of a unique NMDA receptor complex in MSNs with very slow deactivation kinetics.

INTRODUCTION

Synaptic excitation of striatal medium spiny neurons (MSNs) plays an essential role in the physiology of the basal ganglia (Graybiel 2005; Smith and Bolam 1990; Yin and Knowlton 2006). Identifying the synaptic components of striatal excitatory projections may have important implications for understanding various basal ganglia disorders. Excitatory cortical and thalamic inputs to MSNs use N-methyl-D-aspartate (NMDA) receptors (Kita 1996). Biophysical characteristics of NMDA receptors depend on the heteromeric assembly of several gene products (Cull-Candy and Leszkiewicz 2004; Cull-Candy et al. 2001; McBain and Mayer 1994). The subunits assemble in a quaternary array to form functional channels with the majority of receptors being composed of members from the NR1 and NR2 gene families in heterodimeric and heterotrimeric complexes (Kohr 2006). The NR2 subunit family is comprised of four members, NR2A through NR2D, that are expressed in a spatial-temporal fashion throughout the CNS (Cull-Candy and Leszkiewicz 2004; Cull-Candy et al. 2001).

Although the developmentally regulated expression of NR2 subunits in the striatum is observed as in the rest of the brain (Monyer et al. 1994), the NR2B subunit is predominant compared with the NR2A subunit both during development and throughout adult life in rodents (Dunah and Standaert 2003; Lau et al. 2003; Standaert et al. 1994). Furthermore, studies of functional properties of striatal NMDA receptors at excitatory synapses (Chapman et al. 2003; Colwell et al. 1998; Winkler et al. 2004) suggest changes occur around the end of the second postnatal week. At this time, rodents undergo major alterations in striatal anatomy and physiology including synapse, dendritic spine formation (Tepper et al. 1998) and a shift in synaptic plasticity from long-term potentiation (LTP) to long-term depression (LTD) in the dorsolateral striatum (Partridge et al. 2000).

The functional significance of changes in receptor composition is observed in the dorsolateral striatum where the relative expression of NR2A subunit mRNA is greater at later stages compared with the medial striatum (Ganguly and Keefe 2001; Watanabe et al. 1992, 1993). Comparing the kinetics of NMDA–excitatory postsynaptic currents (EPSCs), Chapman et al. (2003) reported a faster rate of decay in MSNs from the dorsolateral compared with ventromedial striatum in rats. A pharmacological analysis of these currents revealed that pure NR1/NR2A and NR1/NR2B heterodimeric assemblies were only partially involved in synaptic transmission and that their relative synaptic contribution changed during development. Furthermore, ifenprodil, the specific NR1/NR2B blocker, failed to change the slow decay kinetics of the NMDA–EPSCs recorded in mouse MSNs (Li et al. 2003, 2004), suggesting the presence of heterotrimERIC NR1/NR2A/NR2B receptor assemblies. All MSNs express NR2A and NR2B subunits (Albers et al. 1999) as part of NR1/NR2A and NR1/NR2B heterodimeric receptors while heterotrimERIC NR1/NR2A/NR2B assemblies predominate at synapses (Dunah and Standaert 2003).

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* S. M. Logan and J. G. Partridge contributed equally to this work.

Address for reprint requests and other correspondence: S. Vicini, Dept. of Physiology and Biophysics, BSB225, Georgetown University School of Medicine, 3900 Reservoir Rd., Washington, DC 20007 (E-mail: stefano.vicini@georgetown.edu).
Despite the critical role of the striatum in regulating the output of the basal ganglia (Bolam et al. 2000; Graybiel 2005; Yin and Knowlton 2006), the characterization of NMDA–EPSCs at excitatory MSN synapses has not been fully examined. In this study, we assess the role of distinct NR2 subunits of the NMDA receptor in striatal excitatory synaptic function using whole cell patch-clamp recordings of excitatory synaptic currents in dorsolateral MSNs in slices prepared from P6-8 and >P12 mice. The decay of the NMDA–EPSC in MSNs was often characterized by the presence of a very slow exponential component. This slow component persisted in MSNs from mice lacking the NR2A or the NR2C subunits, and it was more prevalent in NR2A —/— mice. A further characterization of the NMDA–EPSCs with subunit selective pharmacological agents indicated that the slow component was most likely mediated by an NMDA receptor complex containing the NR2D subunit in either a heterodimeric or -trimeric configuration. A low-affinity competitive NMDA receptor antagonist used to reveal glutamate spillover (Diamond 2001) did not alter the kinetic property of this current. Our results are consistent with the notion that MSNs express multiple NMDA receptor complexes including those containing the NR2D subtype at distinct synaptic and/or extrasynaptic sites and that the NR2A subunit may regulate their expression at these sites.

**METHODS**

Coronal slices (250–300 µm) were prepared from P6–20 C57Bl6J and NR2A —/— or NR2C —/— mice in a C57Bl6J background. The generation of NR2A —/— (e1) mice is reported in Sakimura et al. (1995) and that of NR2C —/— (e3) mice is in Lu et al. (2006) and Karavanova et al. (2007). Mouse pups were killed by decapitation in agreement with the guidelines of the AVMA Panel on Euthanasia and the Georgetown University Animal Care and Use Committee. The whole brain was removed and placed in an ice-cold slicing solution containing (in mM) 85 NaCl, 2.5 KCl, 1 CaCl₂, 4 MgCl₂, 1 NaHPO₄, 25 NaHCO₃, 25 glucose, and 75 sucrose (all from Sigma, St. Louis, MO); pH 7.4 when continuously bubbled with 95% O₂—5% CO₂. Coronal and sagittal slices, including the neostriatum and neocortex, were prepared using a Vibratome 3000 Plus sectioning system (Vibratome, St Louis, MO) and were incubated in the slicing solution at 32°C for 30 min. Slices then recovered for 30 min at 32°C in carbogen-bubbled artificial cerebrospinal fluid (ACSF, in mM): 120 NaCl, 3.1 KCl, 1.25 NaHPO₄, 26 NaHCO₃, 5.0 dextrose, 1.0 MgCl₂, 2.0 CaCl₂, and 0.010 t-serine, 305 mosM, pH 7.4. During experiments, slices were submerged and continuously perfused (2–3 ml/min) with carbogen-bubbled ACSF at room temperature, 22–24°C. Cells were visualized with an upright microscope (Axioscope, Zeiss Germany) using infrared-differential interference contrast video microscopy. Patch pipettes were filled with (in mM) 145 K-gluconate, 1.1 EGTA, 5.0 MgATP, 0.2 Na-GTP, and 10 HEPES to pH 7.2 with KOH supplemented with lidocaine -ethyl bromide (QX314; 4 mM) and 100 HEPES to pH 7.2 with KOH supplemented with lidocaine -ethyl bromide (QX314; 4 mM) for input-output experiments. Alternatively a solution containing (in mM) 120 cesium methanesulfonate, 5 NaCl, 10 tetraethylammonium chloride, 10 HEPEs, 4 QX314, 1.1 EGTA, 5 MgATP, and 0.2 NaGTP was used with pH adjusted to 7.25 using CsOH. Square-wave electric pulses of 100- to 300-µA intensity, 50-µs duration, and 0.05- to 0.1-Hz frequency were delivered by placement of bipolar stimulating electrodes in proximity of the cortico-striatal border. For input-output stimulation experiments (Figs. 5 and 6) intensity was increased ≥5,000 µA. In all experiments, 25 µM bicuculline methiodobromide (BMR, Sigma) was included in the medium to exclude potential contamination of the evoked synaptic response by γ-aminobutyric acid (GABA)-mediated inhibitory synapses. Stock solutions of BMR, 3-[(±)-2-carboxy-2-pyridinyl-4-yl]-propyl-1-phosphonic acid (CPP, Tocris Cookson, Ballwin MO), and 2,3-dihydro-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX, Tocris), d-α-amino adipate (d-AA, Sigma), spermine and ifenprodil (Sigma) were dissolved in water. CP101,606 [(1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol, a gift from Dr. Richard Woodward, Acea Pharmaceutical, Irvine, CA] was dissolved in dimethylsulfoxide (DMSO, <0.1% final concentration). In some experiments, drugs were delivered locally with a Y-tube device (Murase et al. 1989). Stock solutions of BMR, d-AA (both from Sigma), and NBQX, CPP, [±]-cis-1-[phenanthren-2-yl]-carbonyl]piperazine-2,3-dicarboxylic acid (PPDA), and ifenprodil (Tocris Cookson) were dissolved in water. CP101,606 [(1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)—1-propanol, a gift from Dr. Richard Woodward, Acea Pharmaceutical] was dissolved in DMSO (<0.1% final concentration). NMDA–EPSCs were recorded in the presence of 5 µM NBQX (except for experiments in Fig. 2) to analyze the NMDA/non-NMDA ratio.

Patch electrodes (5–7 MΩ) were pulled (Narishige, PP-83, Tokyo, Japan) from borosilicate glass capillaries (Drummond, Broomall, PA). Series resistance (10–15 MD), in whole cell configuration, was monitored for constancy throughout the experiment, and it was not compensated. Current and voltage signals at the headstage of the patch-clamp amplifier (Axopatch 1D, Axon Instrument, Union City, CA) were filtered at 2 kHz, digitized at 5–10 kHz using an IBM-compatible microcomputer equipped with Digitdata 1322A data acquisition board and pCLAMP9 software (both from Molecular Devices, Sunnyvale, CA). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 ( Molecular Devices) software. Spontaneous synaptic currents were identified using a semi-automated template based event detection software (Clampfit 9). Spontaneous NMDA–EPSC averages were based on ≥20 events in each cell studied. The decay phase of currents was fitted using a simplex algorithm for least squares exponential fitting routines with triple exponential equation of the form I(t) = I₁ exp(−t/τ₁) + I₂ exp(−t/τ₂) + I₃ exp(−t/τ₃), where Iᵢ is a peak amplitude of a decay component and τᵢ is the corresponding decay time constant. To allow for easier comparison of decay times between experimental conditions, the three decay time components were combined into a weighted time constant T_w = I_1(1/x_1 + 1/x_2 + 1/x_3) * τ_1 + I_2(1/x_1 + 1/x_2 + 1/x_3) * τ_2 + I_3(1/x_1 + 1/x_2 + 1/x_3) * τ_3. All data values in the text and in the figures are expressed as means ± SE unless otherwise indicated. P values represent the Student’s t-test with at least P < 0.05 as significance threshold.

**RESULTS**

Recordings from striatal neurons in slices

Striatal neurons were visually localized in the dorsolateral caudate putamen of cortico-striatal slices (Fig. 1A). MSNs were identified both by cell size and on the basis of their respective in vitro electrophysiological properties (Fig. 1B). These characteristics were used to distinguish MSNs from a small minority of putative cholinergic and/or GABAergic interneurons that have distinct size and action potential firing patterns (Bennett and Wilson 1999; Kawaguchi 1993; Kreitzer and Malenka 2007; Shen et al. 2005). Figure 1B shows the membrane potential response of a typical MSN recorded in current-clamp mode on injecting a sequence of hyper- and depolarizing currents. The records demonstrate a repetitive and nonadapting spike-firing pattern typically reported for MSNs in response to depolarizing current injection. In addition, the records also show a decrease in resistance in response to hyperpolarizing current injections due to the presence of an inward rectifier potassium current (Kita et al. 1984; Shen et al.
Excitatory synaptic currents from striatal neurons

Striatal neurons express both non-NMDA- and NMDA-type ionotropic glutamate receptors (Dunah and Standaert 2003; Kita 1996; Standaert et al. 1994). Afferent stimulation releases glutamate from presynaptic boutons, which then activates receptors on postsynaptic striatal neurons producing an EPSC. We utilized whole cell voltage-clamp recordings to examine the EPSCs from striatal neurons at a holding voltage of −70 mV using pharmacological agents to isolate either the non-NMDA- or NMDA-mediated responses. These data were gathered in wild-type mice and in mice lacking the NR2A or the NR2C subunit of NMDA receptors. The average responses shown in Fig. 2A were recorded from MSNs in wild-type mice at P6 and P13 in the absence of Mg2+ to attain the NMDA-mediated component of the EPSC (NMDA–EPSC). The GABA_A receptor blocker, BMR (25 µM) was also included to eliminate any contaminating GABA receptor-mediated current. In many cells examined under these conditions, we observed a long-lasting synaptic current that was completely blocked by the nonselective NMDA receptor antagonist, CPP (10 µM; Fig. 2, A and B). The evoked current remaining in the presence of 10 µM CPP was much more rapid in offset and represents current carried by non-NMDA-type glutamate receptors (non-NMDA–EPSC). Using this approach, we characterized the non-NMDA- and NMDA–EPSCs from mice at two distinct developmental age groups; P6–P8 and greater than P12 (P13–P20). These time points were selected because they bracket the period around eye opening, potentially a critical indicator for the beginning of the maturation of motor coordination. It has to be considered, however, that the P13–P20 group is still undergoing developmental maturation and further changes may occur in adult life. The peak amplitudes of the NMDA- and non-NMDA–EPSCs (NMDA/non-NMDA ratio, Fig. 2C), recorded from MSNs in young (P6–8) wild-type animals, show that the NMDA component is approximately twofold greater than the non-NMDA-mediated response. In contrast, the NMDA and non-NMDA components contribute to the EPSC equally in cells from the older age group (>P12). Mice lacking the NR2A subunit, on the other hand, show NMDA/non-NMDA ratios that are significantly smaller compared with

FIG. 1. Medium spiny neurons (MSNs) can be identified by firing pattern. A: photomicrograph of a mouse corticostriatal slice illustrating the position of recording (right) and stimulating (left) electrodes. Scale bar = 500 µm. The white asterisk illustrates the cortical stimulation site described in Figs. 5 and 6. B: superimposed membrane potential responses of a striatal neuron in response to hyperpolarizing and depolarizing current injections (bottom) in current-clamp mode. The records demonstrate the typical inward rectification and spike firing pattern reported for MSNs.

FIG. 2. The contribution of the N-methyl-D-aspartate (NMDA) receptor to synaptic currents in MSNs. A and B: superimposed current traces from wild-type (A) and NR2A −/− (B) MSNs at P6 (left) and P13 (right) in the absence of Mg2+. The black traces are the average of 15 traces in the presence of 25 µM bicusculine methiobromide (BMR), representing the combined non-NMDA and NMDA response (T1). The dashed traces are the average EPSCs evoked in the presence of 10 µM 3-(±)-2-carboxypiperazine-4-yl-propyl-1-phosphonic acid (CPP) to block NMDA receptors, representing the non-NMDA-mediated responses (T2). Shown in the gray traces (T1–T2) are the digital subtraction of the dashed traces (non-NMDA–EPSC) from the black traces (total EPSC). These show the average synaptic response mediated by NMDA receptors alone (NMDA–EPSC). Scale bar applies to all traces. C: summary of the ratio of the peak amplitude of the NMDA–EPSC to that of the non-NMDA–EPSC (NMDA/non-NMDA ratio) measured from MSNs (cell number above each bar) in wild-type (wt) mice and in mice with a deletion of either the NR2A (NR2A −/−) or the NR2C (NR2C −/−) subunit. The NMDA/non-NMDA ratio decreased with development and was significantly less between age-matched wild-type and NR2A −/− mice. Data are derived from ≥3 mice grouped at postnatal days 6–8 (P6) and postnatal days 12–20 (>P12). *P < 0.05 significant to the group at age > P12. +P < 0.05 significant to wild type.
Long-lasting decay of NMDA–EPSCs in striatal neurons

The striking feature of NMDA–EPSCs in all groups tested was the long-lasting decay phase. The sum of two or three exponential curves was usually needed to best fit the decay of evoked NMDA–EPSCs recorded in the presence of 5 μM NBQX. Table 1 summarizes the values of the three time constants used for the exponential fitting and their relative contribution to peak amplitude for the experimental groups. To allow comparison between groups, we used a weighted time constant (Tw) to describe the time course of the synaptic currents. We found that the Tw of evoked NMDA–EPSCs became shorter in older, wild-type MSNs. Tw’s did not show any significant change with development in MSNs from NR2A −/− mice, and they were twice as long in MSNs from wild-type mice. The decay of NMDA–EPSCs was consistently slowed down by the occurrence of a very slow time constant (>1 s) that appeared with variable proportion in all the different groups (Table 1). When the experimental group >P12 (n = 59) was further subdivided in a group at P13–P16 (n = 43) and a group at P17–P20 (n = 14), no significant differences were observed and the results were lumped together. A fitting-independent assessment of the NMDA–EPSCs duration, achieved by dividing the area of the current by the peak amplitude, yielded comparable results (data not shown).

A long-lasting decay in the synaptic current may be related to poor-quality voltage and space clamp of excitatory synapses on elaborated dendritic spines in MSNs. To reduce space-clamp errors, we used a cesium-based intracellular solution that blocks most potassium channels. This approach results in a better estimate of EPSCs in MSNs as discussed by Day et al. (2006). Cesium-based internal solution did not change the characteristics of the NMDA–EPSC, suggesting that voltage-clamp control is less likely to be a source for the slow-down of the response (n > 4 in each experimental group). Poor synchronization of presynaptic action potentials in distinct excitatory afferents may be another explanation for long-lasting components of synaptic currents observed in MSNs. To address this, we took advantage of a few cells in the group at P6–8 where spontaneous NMDA–EPSCs (sNMDA–EPSCs) occurred between subsequent evoked NMDA–EPSCs (Fig. 3A). As shown in the example in Fig. 3, the average sNMDA–EPSC (Fig. 3B, left) has a Tw that is very similar to that attained for the average evoked NMDA–EPSC in the same cell (Fig. 3B, right). Similar results were observed in eight MSNs studied in the group at P6–P8 that displayed sNMDA–EPSCs (Fig. 3C). These spontaneous currents were likely due to release from single presynaptic sites as shown in Tang et al. (2001) for s-non-NMDA–EPSCs. These results suggest that MSNs likely possess an NMDA receptor complement that yields NMDA–EPSC decay components with a long decay constant that is activated by synaptic release of neurotransmitter.

Action of NR2B antagonists on NMDA-EPSCs from striatal neurons

To further characterize the NMDA mediated synaptic current, we used the noncompetitive, NR2B-selective antagonist, ifenprodil (10 μM) and its derivative CP101,606 (CP, 10 μM, Mott et al. 1998). Ifenprodil acts in a use-dependent and voltage-independent manner and demonstrates a >400-fold IC50 for currents expressed from NR1/NR2B compared with those expressed from NR1/NR2A heterodimers (Berberich et al. 2005; Kew et al. 1998; Kohr 2006). Ifenprodil derivative, CP also shows a maximal inhibition that is greatly reduced when currents are expressed from NR1/NR2A/NR2B triheteromers (Hatton and Paoletti 2005). When recording evoked currents from MSNs, a portion of the NMDA-mediated response was blocked in the presence of either antagonist, whereas the long-lasting component persisted. An example of this blockade is shown in Fig. 4A in which the evoked response of a MSN is shown in the presence and absence of CP (note the

<table>
<thead>
<tr>
<th>Group</th>
<th>Tw</th>
<th>Percentage Tw</th>
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<tbody>
<tr>
<td>+/+ (P6–8)</td>
<td>161 ± 8</td>
<td>48.4 ± 6</td>
</tr>
<tr>
<td>2A −/− (P6–8)</td>
<td>115 ± 7</td>
<td>218 ± 9</td>
</tr>
<tr>
<td>2C −/− (P6–8)</td>
<td>82 ± 11</td>
<td>173 ± 12</td>
</tr>
<tr>
<td>+/+ (P12)</td>
<td>238 ± 43</td>
<td>445 ± 57</td>
</tr>
<tr>
<td>2A −/− (P12)</td>
<td>47 ± 20</td>
<td>212 ± 72</td>
</tr>
</tbody>
</table>

Percentage Tw is the percent of cells with the slowest exponential component (>1,000 ms). *P < 0.01 vs. wild type at the same postnatal day; †P < 0.05 vs. P6–8 within genotype.
CP insensitive
spontaneous

CP that is significantly different from the reduction seen at P12
MSNs recorded in wild-type mice at P6–P8 show a 50%
animals at earlier stages of development in the presence of CP. We obtained when currents were recorded in MSNs from
NR2B blockade. This interpretation is consistent with the data
subtypes in the synaptic response that are less sensitive to
other genotypes. Our data may indicate that a compensatory
predicted that the NMDA-EPSC from these knockout animals
NR1/NR2B receptors. If such was the case, we would have
NR2A
mice; this might be expected to express mostly
NR2C types are thought to predominate in receptor complexes at
Thus even early in development, when NR2B receptor sub-
types are thought to predominate in receptor complexes at
synapses (Monyer et al. 1994), the NR2A −/− MSNs show a
reduced NR1/NR2B receptor complement again, suggesting
that the presence of the NR2A subunit is important in regulat-
ing the NMDA receptor composition at synapses.

We then analyzed the kinetics of the evoked current in the
presence and absence of CP (Fig. 4A). In the majority of cells, the
NMDA–EPSCs became slower and smaller in the presence
of the NR2B blocker (denoted as the CP-insensitive current in
Fig. 4), an effect evidenced in all genotypes examined. Similar
results were seen with ifenprodil (not shown). Subtraction of
the CP-insensitive current from the total evoked current reveals
the CP-sensitive component of the NMDA–EPSC (Fig. 4A).
An analysis of the decay phase of the CP-sensitive current
yield Tw values in the range of 250–300 ms consistent with the

FIG. 4. The long-lasting NMDA receptor-mediated excitatory postsynaptic
current (EPSC) is not blocked by an NR2B antagonist. A, top: superimposed
current traces at −70 mV holding potential from a wild-type mouse MSN at
P14 in the absence of Mg2+. The black trace is averaged NMDA-EPSC in the
presence of 25 μM BMR and 5 μM NBQX. The gray trace is the average
NMDA-EPSC evoked in the presence of 10 μM CP101,606 to block NMDA
receptors containing NR2B. Bottom left: Shown in isolation is the CP-
insensitive component of the response (a) superimposed with a double-
exponential fit to the decay process. The Tw of this fit curve is indicated above
the trace. Bottom right: digital subtraction of the 2 traces results in the CP
sensitive component (b) again superimposed with a double-exponential fit. The
Tw is indicated above the trace. Dotted lines are the baseline currents.

FIG. 3. Evoked and spontaneous NMDA-EPSCs demonstrate similar decay
rates with slow kinetics. A: representative recording from an MSN at P6
illustrating spontaneous excitatory postsynaptic currents mediated by NMDA
receptors (sNMDA-EPSCs) recorded in a Mg2+-free solution with 25 μM
BMR and 5 μM NBQX. sNMDA-EPSCs occurred spontaneously between
responses evoked at 0.07 Hz. Holding potential was −70 mV. B: average
sNMDA-EPSC (left) is compared with the average evoked NMDA-EPSC
(right) recorded from the same neuron as in A. An indication of the weighted
time constant of decay (Tw) is provided below each trace. C: summary of the
Tws of averaged sNMDA-EPSC compared with those of the average evoked
NMDA-EPSC in MSN from wild-type mice at P6–P8. Data derived from 8
cells in 3 mice.

Tw (ms)

0 200 400 600 800

□ evoked □ spontaneous

Tw 491 ms

B

A

C

an in every genotype studied. More importantly, CP blockade of
NMDA-EPSCs was actually greater in wild-type MSNs at
P6–P8 than that measured in age-matched NR2A −/− mice
(31 ± 5%, n = 9) but not in NR2C −/− (52 ± 2%, n = 6).
Thus even early in development, when NR2B receptor sub-
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A

C

an in every genotype studied. More importantly, CP blockade of
NMDA-EPSCs was actually greater in wild-type MSNs at
P6–P8 than that measured in age-matched NR2A −/− mice
(31 ± 5%, n = 9) but not in NR2C −/− (52 ± 2%, n = 6).
Thus even early in development, when NR2B receptor sub-
types are thought to predominate in receptor complexes at
synapses (Monyer et al. 1994), the NR2A −/− MSNs show a
reduced NR1/NR2B receptor complement again, suggesting
that the presence of the NR2A subunit is important in regulat-
ing the NMDA receptor composition at synapses.

FIG. 3. Evoked and spontaneous NMDA-EPSCs demonstrate similar decay
rates with slow kinetics. A: representative recording from an MSN at P6
illustrating spontaneous excitatory postsynaptic currents mediated by NMDA
receptors (sNMDA-EPSCs) recorded in a Mg2+-free solution with 25 μM
BMR and 5 μM NBQX. sNMDA-EPSCs occurred spontaneously between
responses evoked at 0.07 Hz. Holding potential was −70 mV. B: average
sNMDA-EPSC (left) is compared with the average evoked NMDA-EPSC
(right) recorded from the same neuron as in A. An indication of the weighted
time constant of decay (Tw) is provided below each trace. C: summary of the
Tws of averaged sNMDA-EPSC compared with those of the average evoked
NMDA-EPSC in MSN from wild-type mice at P6–P8. Data derived from 8
cells in 3 mice.
deactivation kinetics of currents derived from heterologous cells expressing NR1/NR2B heteromers (Vicini et al. 1998). Such findings indicate that NR2B subunits are most likely present at synapses and participate in the evoked currents of MSNs. Thus the CP-sensitive NMDA receptors may either be in the form of NR1/NR2B heterodimers or in a heterotrimeric form comprising another NR2 subunit that would necessarily have some degree of sensitivity to the NR2B blocker (Hatton and Paolotti 2005). The Tws of the CP-insensitive currents were not significantly different between genotypes at the time points examined (Fig. 4B, black bars). Thus MSNs from mice lacking NR2A receptors or NR2C receptors show currents with a long-lasting decay that share similar characteristics with those of currents derived from the wild-type mice. Such a finding suggests that the long-lasting component of the NMDA–EPSC is not likely mediated by NR1/NR2A or NR1/NR2D heteromeric assemblies at synapses. The Tw of the CP-insensitive component has a threefold longer time course than that of the CP-sensitive component in most cells. The only NMDA receptor complex shown by recombinant studies to possess this long deactivation kinetics are those containing the NR2D subunit (Monyer et al. 1994; Vicini et al. 1998). Thus the long decay of evoked currents from MSNs may be mediated by NR1/NR2B/NR2D heterotrimeric assemblies or NR1/NR2D complexes that could participate in evoked NMDA–EPSCs possibly through diffusional transmission by glutamate spillover. As reported in Lozovaya et al. (2004), we used PPDA, a competitive antagonist moderately selective for NR2 subunits (Feng et al. 2004; Hrabetova et al. 2000), to further dissect the contribution of this subunit to striatal NMDA–EPSCs. In three MSNs from wild-type mice (> P12) and two MSNs from NR2AKO (P6), 10 μM PPDA completely abolished the NMDA–EPSCs in striking contrast to the lack of action of this drug on hippocampal NMDA–EPSCs (Lozovaya et al. 2004). Lower doses of this compound reduced the peak NMDA–EPSCs in wild-type MSNs (P > 12) by 76 ± 3% (2 μM, n = 6) and 14 ± 8% (0.3 μM, n = 6). No changes in decay kinetics were observed at any PPDA dose tested.

Relative excitability of MSNs is mediated by NMDA receptors and depends on stimulus intensity

The work of Kita (1996) demonstrated that increasing stimulus intensity and frequency of excitatory afferents to MSNs elicited larger EPSPs. The CPP sensitivity of a component of these potentials suggested an increasing contribution of NMDA receptors to the postsynaptic responses depending on the conditions of stimulation. Thus we investigated the dependence of the stimulus location and intensity of the NMDA–EPSCs elicited in striatal neurons while recording in voltage-clamp mode. These experiments showed that the level of stimulus intensity required to elicit a response was different for distinct stimulus locations. Figure 5A shows examples of these responses with NMDA–EPSCs increasing in amplitude with increasing stimulus intensity. As MSNs receive both thalamic and cortical inputs, we also considered the location of the stimulation electrode. When the stimulating electrode was placed in an intracortical location, an area that should activate selected cortical afferents, we observed that all of the MSNs tested required a high-intensity stimulus to evoked NMDA–EPSCs (n = 8 of 8 cells). As such, we have categorized these cells as “high threshold” in our analysis. In contrast, placement of the stimulating electrode either in proximity to the corticostriatal border or in an intrastriatal location, an area that stimulates mixed (thalamic, cortical and striatal) afferents elicited NMDA–EPSCs with a stimulation intensity one order of magnitude lower than that required in the intracortical location (n = 9 of 11 cells). Consequently, we have categorized these cells as being “low threshold.” Such findings suggest that stimulus threshold is correlated with the location of the stimulating electrode; afferents from the cortex require a higher level of intensity for recruitment, whereas a response from intrastriatal fibers requires a lower stimulus threshold for activation. Our classification scheme does not imply the existence of two separate types of MSNs but simply correlates with the stimulus intensity used to produce the NMDA–EPSCs from different locations. This does not exclude the possibility that the same MSN may yield NMDA–EPSCs that differ depending on the stimulus location used.

A comparison of the average NMDA–EPSCs obtained from high- and low-threshold MSNs showed that although the current amplitude did not differ, the decay phase was considerably altered; low-threshold MSNs showed Tw values that were slower than those obtained from the high-threshold cells. This is demonstrated in Fig. 5B, which illustrates the overlay of responses from various stimulus intensities normalized to the maximal peak amplitude attained for a particular cell. Using a
stimulus intensity half that needed to attain a maximal response for a given cell, we were able to combine results from different cells with varying thresholds. On average, the weighted time constant of the evoked EPSCs near the half-maximal response amplitude was $338 \pm 33$ ms in low-threshold neurons compared with $206 \pm 28$ ms in high-threshold neurons ($P < 0.05$, t-test). Figure 5, C and D, illustrates, on a logarithmic scale, the dependence of the averaged amplitude and Tws of all cells studied as a function of stimulation intensity. A consistent amplitude increase was observed when the stimulus intensity was increased in both high- and low-threshold MSNs (Fig. 5C, left). Although stimulus intensity-dependent increase in Tws was observed in both cell types, this was slightly more common for the low-threshold than for high-threshold intensity cells (Fig. 5D). We also investigated stimulus-dependent changes in the NMDA:non-NMDA ratio at both stimulus locations. In seven neurons of wild-type >P12 experimental groups, we determined that the ratio of the two components does not change significantly with stimulus intensity similar to what is reported in Li et al. (2004).

As the orientation of the section plane to make coronal slices affects the ability to stimulate different afferents to MSNs, we investigated stimulus location in sagittal slices. The Tw of the evoked EPSCs near the half-maximal response amplitude was 1.51 times slower for low-threshold neurons ($n = 6$, in 3 mice at $P > 12$) compared with high-threshold neurons in sagittal slices. This value averaged 1.64 for coronal slices.

Long decay phase of NMDA–EPSCs from striatal neurons is not likely caused by glutamate spillover

The difference in decay of NMDA–EPSCs evoked by the two stimulus locations could exist as a result of distinct subtypes of NMDA receptors and/or the occurrence of volume transmission by spillover of glutamate onto receptor subtypes outside of the synapse (for review, see Kohr 2006; Sem’yanov 2005). Volume transmission has been defined as glutamate that escapes the synaptic cleft “spilling over” onto extrasynaptic targets and neighboring synapses (Diamond 2001). To investigate the involvement of spillover in the generation of synaptic currents with long-lasting decay, we stimulated MSNs from both locations using brief trains of high-frequency stimuli ($7 \times 100$ Hz), a protocol that has been shown elsewhere to generate spillover at excitatory synapses (Lozovaya et al. 2004). We then used this technique to elicit NMDA–EPSCs from both high- and low-threshold MSNs and compared them to those evoked using a single stimulus (Fig. 6A and B). As before, the stimulus intensity given was half that needed to attain a maximal response for a given cell and produced NMDA–EPSCs comparable to those in previous experiments. The results from these experiments show that both the amplitude of the current and the Tws were increased considerably for the low- and high-threshold MSNs when the brief train stimulus protocol was used (Fig. 6C). Such findings suggest that, under high-frequency stimulation, glutamate may be released faster than it can be removed from the synapse by transporters and

![FIG. 6. The impact of stimulation frequency and intensity on the NMDA-EPSC decay. A and B: comparison of averaged NMDA-EPSCs elicited by single stimuli (black traces) and brief, high-frequency trains of stimuli ($7 \times 100$ Hz, gray traces) in a “low-threshold” MSN (A) and a “high-threshold” MSN (B) as defined in Fig. 5. C: bar graph summary of the percent changes elicited by a brief train of presynaptic stimuli on NMDA-EPSC amplitude (left) and decay (right). D: superimposed averaged NMDA-EPSCs elicited by brief trains of stimuli in the absence (black, basal HFS) and presence of 10 $\mu$M CP101,606 (gray, CP HFS). Right: 2 traces are shown superimposed and normalized to peak current amplitude.](http://jn.physiology.org/)

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thus may act on receptor complexes that are perisynaptically and/or extrasynaptically located (Kohr 2006; Semyanov 2005). As suggested by Lozovaya et al. (2004), the receptor complexes at these locations may include the NR2D subunit expressed either as an NR1/NR2D heterodimer or as a heterotrimer in combination with an NR2B subunit as previously discussed. This proposal was supported by the sensitivity to an NR2B blocker. Thus we studied the NMDA–EPSCs evoked by brief trains of high-frequency stimuli in the presence of CP (Fig. 6). Because we observed no difference in the percent increase of the peak current or the Tw (Fig. 6C), we combined data in which high-frequency stimulation was given to cells characterized as either high or low stimulus threshold (n = 6). The peak amplitude of the NMDA–EPSCs recorded following a 7 × 100-Hz HFS protocol decreased by 42 ± 4% in the presence of CP (P < 0.01, 2-tailed t-test) and the Tw increased by 48 ± 12% (P = 0.01, n = 5, Fig. 6D). In addition, the percent contribution of the fast kinetic component to peak amplitude decreased from 88 ± 3% under control HFS conditions to 59 ± 11% in HFS plus CP (P < 0.05). Thus in response to high-frequency trains, the CP insensitive component was slower than the CP-sensitive one.

These results together with the stimulus intensity-dependent increase in the decay times (Fig. 5D) suggest that a stronger stimulus may induce glutamate spillover allowing perisynaptic and/or extrasynaptic receptors to participate in the synaptic response. To further investigate the possibility of spillover, we studied the effects of the low-affinity NMDA receptor antagonist, D-AA (70 μM), on evoked NMDA–EPSCs while holding the cell at −70 mV. Spillover would cause the persistence of a low concentration of glutamate outside the synapse participating in the NMDA–EPSCs. A low-affinity, competitive antagonist of the NMDA receptor, such as D-AA, would antagonize these low glutamate concentration tails to a greater extent than the high concentration in the cleft that occurs during the synaptic response (Diamond 2001). Acute application of D-AA decreased the amplitude of NMDA–EPSCs evoked at half-maximal stimulus by 62 ± 7% (P < 0.01). In the six neurons of wild-type mice at P > 12, the Tw averaged 257 ± 23 ms before D-AA application, whereas the Tw averaged 291 ± 22 ms in the presence of the antagonist (P > 0.30, t-test). Further separation of cells in this experimental group into high- and low-threshold subgroups (3 cells each) did not reveal any significant difference from the whole experimental group taken together. We then compared the action of D-AA on NMDA–EPSC elicited by brief trains. In seven cells of mice at P > 12, D-AA reduced the peak amplitude to an extent comparable to that observed with single stimuli but also failed to alter the NMDA–EPSC decay. Taken together such findings support a synaptic localization of NMDA receptors that exhibits slow decay kinetics.

DISCUSSION

We investigated the developmental expression of NMDA receptor subunits at striatal synapses using whole cell patch-clamp recordings of excitatory postsynaptic currents from dorsolateral medium spiny neurons. By extension of previous findings (Chapman et al. 2003; Li et al. 2003, 2004), we used P6-8 and >P12 mice to examine the properties of NMDA receptors in wild-type MSNs in addition to mice of similar ages that were lacking either the NR2A or the NR2C subunit. Our findings show that the ratio of the NMDA to non-NMDA component of EPSCs decreases with development. Furthermore, this ratio is significantly reduced between age-matched +/+ and NR2A −/− neurons. Some of the differences observed in NMDA/non-NMDA ratio between genotype may relate to changes in channel properties of receptors with distinct subunit composition. However, our observations are in agreement with findings from other brain regions where the loss of NMDA receptor-rich silent synapses have been shown to yield similar changes in synaptic currents (Kullmann 2003). In contrast, no difference in the NMDA to non-NMDA ratio was observed between aged-matched +/+ and NR2C −/− mice, suggesting that the NR2C subunit plays little role in determining synaptic NMDA receptors. Such findings are consistent with anatomical studies showing low abundance of this subunit in the striatum (Dunah and Standaert 2003; Lau et al. 2003; Standaert et al. 1994).

In many MSNs, the decay phase of the NMDA–EPSC decay was characterized by the presence of up to three distinct exponential components including a very slow component with a time constant lasting >1 s regardless of genotype or age. The contribution of this component was quite variable and depended on stimulation intensity, frequency, and location. Our use of a cesium-based intracellular solution to minimize space-clamp distortions and the similar decay seen in spontaneous events suggest that the origin of this slow component is not likely due to poor voltage control of the dendrite or to lack of synchronization between distinct monosynaptic afferents. This slow time constant contributed to a much greater degree in NR2A −/− mice compared with age-matched controls. In addition brief trains of presynaptic stimuli prolonged the duration of the NMDA–EPSCs. Combined, these findings are consistent with the notion that MSNs express multiple receptor complexes at distinct synaptic and/or extrasynaptic sites and that the NR2A subunit may regulate the expression of receptors at these sites.

NMDA receptor subunits shape NMDA-EPSCs in MSNs

We see a significant developmental decrease in the decay of NMDA-EPSCs (from 599 ± 23 to 401 ± 34 ms) and a mild decline in the portion of current blocked by the NR2B antagonist (CP), at later developmental stages (>P12) in wild-type mice. These results are entirely consistent with those reported previously (Chapman et al. 2003) and may reflect the progressive increase in receptor complexes containing the NR2A subunit reported in most regions of the brain (Monyer et al. 1994). No further speed-up in decay time or decrease in CP sensitivity is seen at developmental ages >P12, suggesting that the receptor phenotype is most likely expressed in its mature form at an age corresponding to the period of eye opening in mice. This may reflect a requirement for improved striatal motor control at a developmental time point when visual and motor inputs are being sculpted into a meaningful physiological network.

In contrast to wild-type cells, no developmental decrease in the Tw of the NMDA–EPSCs was observed in MSNs from the NR2A −/− mice, and the fast component of decay of NMDA–EPSCs in neurons from NR2A −/− mice was either absent or remarkably slower compared with age-matched controls (see...
possible role of extrasynaptic NMDA receptors

NMDA receptors are found at both synaptic and extrasynaptic sites. In cerebellar Golgi cells, the presence of the NR2D subunit in NMDA receptor complexes excludes them from synapses (Brickley et al. 2003). In hippocampal neurons, the NR1/NR2B receptor subtype is preferentially located extrasynaptically and mediates cross-talk among synapses (Scimemi et al. 2004). To assess the involvement of such receptor types in the generation of the long-lasting NMDA–EPSC, we altered the number and types of synapses activated by changing the stimulus strength and location. Input-output studies indicated that stimulus location and intensity were correlated. Stimulation from intracortical locations required higher intensities to elicit responses that produced relatively fast NMDA–EPSCs. Stimulation in the striatum more proximal to the recorded cell required lower intensities to elicit responses that exhibited slower NMDA–EPSCs. We found this relation existed in both coronal and sagittal slices. Such findings are not surprising given the high degree of convergence of cortical inputs onto MSNs with a large number of presynaptic axons being required to elicit postsynaptic responses (Zheng and Wilson 2002). Thus a stronger stimulus was needed to excite cortical afferents when the stimulus was placed distally. However, the proximal stimulation site may require a lower threshold to evoke responses in MSNs because the presynaptic axons are recruited from a larger pool of both cortical and thalamic origin.

An important observation from these experiments is that the amplitude and decay time of the response increased progressively with stimulation intensity independently of location. In addition, the decay time was significantly faster in the high-threshold MSNs although the participation of extrasynaptic receptors may also be involved (see following text). Alternatively, however, intrastratial stimulation is known to induce dopaminergic and cholinergic release (Cepeda and Levine 1998, 2006; Partridge et al. 2002), which may result in the modulation of synaptic NMDA receptor function through second-messenger activation.

Our stimulus-frequency experiments were performed in light of studies from CA1 pyramidal neurons in which brief high-frequency presynaptic trains evoked NMDA–EPSCs that were larger in amplitude with slower kinetics than those evoked with a single stimulus (Lozovaya et al. 2004). These findings suggested that extrasynaptic NMDA receptors composed of NR2B and NR2D subunits participated in the evoked response as a result of the high-frequency stimulus they used. More rapid stimuli may lead to a spillover of glutamate unto a population of receptors located in a region outside of the release site. This type of spillover mechanism is thought to participate in glutamatergic transmission to varying degrees (Huang 1998; Kullmann and Asztely 1998) and depends on such factors as the geometry of synapses and the synaptic environment (Sem’yanov 2005; Sykova 2004). Similar to CA1 neurons, we found that the slower component was enhanced by a brief stimulus train in MSNs, suggesting the involvement of glutamate spillover possibly onto extrasynaptic receptors. However,
the application of CP had little effect on the slower component of the evoked response in MSNs and resulted in a slow down of the NMDA–EPSC in contrast to the speed up of NMDA–EPSC seen with ifenprodil in CA1 neurons (Lozovaya et al. 2004). Such results point to an NMDA receptor subtype at MSN synapses such as the NR2D subunit that is not sensitive to CP blockade but that exhibits long decay kinetics (Monyer et al. 1994; Vicini et al. 1998). t-aa, a low-affinity antagonist that has been used to block the participation of extrasynaptic NMDARs in evoked responses (Diamond 2001), failed to affect the kinetics of the slow component whether the stimulus was delivered as a single event or as a train. These observations further support to the hypothesis that receptors at striatal synapses are not activated by volume transmission of glutamate in which the neurotransmitter is released to such an extent that it activates both synaptic and extrasynaptic NMDA receptors proximal and distal to the release site (Diamond 2001). Our observations, therefore argue for a synaptic location of receptors that have intrinsic channel properties yielding long decay currents.

Subunit composition of striatal NMDA receptor complexes

The majority of our evidence supports the existence of an NMDA receptor at MSN synapses that are endowed with long-lasting activation. However, the actual subunit composition of such a receptor subtype is difficult to resolve. Although the NR2B subunit is likely to be present, our findings cannot exclude the possibility that these receptors may be comprised by the NR2D subunit either in part (NR1/NR2B/NR2D) or in full (NR1/NR2D) as the ability of antagonists to distinguish between these receptor subtypes is, at the moment, questionable. The physiological existence of NR1/NR2B/NR2D channels has been recently identified in cerebellar Golgi neurons (Brickley et al. 2003). These studies, in combination with those examining the dopaminergic neurons of the pars compacta (Jones and Gibb 2005), indicate that the NR2D subunit confers to the channel complex a resistance to selective NR1/NR2B subtype blockers such as ifenprodil and CP101,606 (Mott et al. 1998). In fact, although NR1/NR2D- and NR1/NR2B-containing receptors give rise to low (20/40 pS)- and high-conductance events (50 pS), respectively (Cull-Candy et al. 2001), the heterotrimeric assembly (NR1/NR2B/NR2D) generates 50-pS channels lacking sensitivity to ifenprodil (Brickley et al. 2003; Jones and Gibb 2005).

Such findings are consistent with our observations in that CP was unable to block the slow component of the NMDA–EPSC and suggest that this current may be carried by NR1/NR2B/NR2D receptors. The complete blockade of NMDA–EPSCs in MSNs with the nonspecific NR2D antagonist, PPDA, is in striking contrast to the lack of action of this drug on hippocampal NMDA–EPSCs (Lozovaya et al. 2004). If the selectivity of this compound for NR2C- and NR2D-containing NMDA receptor will be verified, it may mean that the majority of receptors at striatal synapses comprise the NR2D subunit. Little difference was observed in the action of PPDA on MSNs from NR2A−/− mice, further supporting the existence of functional NR1/NR2B/NR2D receptors as the mediator of the slow component of NMDA–EPSCs.

At present, no studies have examined the deactivation properties of the heterotrimers in response to the brief glutamate concentration jumps, such as those that occur at synapses. However, an elegant comparative study of membrane patches isolated from distinct neurons of the basal ganglia (Gott et al. 1997) shows that MSNs do not differ from the dopaminergic neurons in terms of deactivation of macroscopic NMDA current. This study further showed that the NMDA channel deactivation was the slowest in these two types of neurons. Such studies provide further support to our suggestion of a functional role for the NR2D subunit in shaping the synaptic responses in individual cell populations of the basal ganglia.

Physiological relevance of slow NMDA-EPSCs

The presence of NMDA receptors may contribute to the general excitability of MSNs because they regulate transition to upstates in vitro (Vergara et al. 2003). With the caveat that we have characterized the long-lasting deactivation kinetics of NMDA-mediated currents in MSNs at room temperature, the heterotrimERIC receptor complex that we propose here may have great importance in determining the intensity and the timing of the excitatory cortical or thalamic activation at physiological temperatures. In addition, because NMDA channels are permeable to calcium ions, the prolonged NMDA–EPSC could lead to excessive calcium loading of MSNs. Calcium loading is implicated in neurotoxicity and thus a long-lasting calcium signal could begin to explain why MSNs are very susceptible to excitotoxic damage (Hardingham and Bading 2003).

In addition to increasing the amplitude and duration of synaptic responses, a long-lasting NMDA channel might be expected to have profound effects on information processing in the striatum. Along these lines, as the rate of spontaneous vesicular glutamate release is >1 Hz in the striatum (Day et al. 2006; Tang et al. 2001), and the deactivation kinetics of NR2D containing channels in response to a single glutamatergic vesicle is >1 s (Misra et al. 2000; Vicini et al. 1998; Wyllie et al. 1998), the normal spontaneous vesicular release would be expected to tonically activate these types of NMDA channels with important functional consequences for striatal excitability.

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Present address of S. M. Logan: Laboratory of Integrative Neuroscience, University of Illinois, Chicago, IL 60607.

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SYNAPTIC NMDA RECEPTORS IN STRIATAL NEURONS


