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Differences in excitatory transmission between thalamic and cortical afferents to single spiny efferent neurons of rat dorsal striatum

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Abstract

The striatum is crucially involved in motor and cognitive function and receives significant glutamate input from cortex and thalamus. The corticostriatal pathway arises from diverse regions of cortex and is thought to provide information to the basal ganglia from which motor actions are selected and modified. The thalamostriatal pathway arises from specific thalamic nuclei and is involved in attention and possibly strategy switching. Despite these fundamental functional differences, direct comparisons of the properties of these pathways are lacking. NMDA receptors at synapses powerfully affect postsynaptic processing, and incorporation of different NR2 subunits into NMDA receptors has profound effects on the pharmacological and biophysical properties of the receptor. Utilization of different NMDA receptors at thalamostriatal and corticostriatal synapses could allow for afferent-specific differences in information processing. We used a novel rat brain slice preparation preserving corticostriatal and thalamostriatal pathways to medium spiny neurons to examine the properties of NMDA receptor-mediated EPSCs recorded using the whole-cell, patch-clamp technique. Within the same neuron, the NMDA/non-NMDA ratio is greater for excitatory responses evoked from the thalamostriatal pathway versus the corticostriatal pathway. In addition, reversal potentials and decay kinetics of the NMDA receptor-mediated EPSCs suggest that the thalamostriatal synapse is more distant on the dendritic arbor. Finally, results obtained with antagonists specific for NR2B-containing NMDA receptors imply that NMDA receptors at corticostriatal synapses contain more NR2B subunits. These synapse-specific differences in NMDA receptor content and pharmacology provide potential differential sites of action for NMDA receptor subtype-specific antagonists proposed for the treatment of Parkinson's disease.

Keywords

NMDA receptor; rat; thalamostriatal; corticostriatal; Parkinsons Disease

Introduction

The basal ganglia (BG) are a group of subcortical nuclei known to be involved in motor planning and execution (Graybiel *et al.*, 1994; Mink, 1996; Graybiel, 1998; Hollerman *et al.*, 2000), reinforcement learning (Houk *et al.*, 1995; Schultz *et al.*, 1997; Packard & Knowlton, 2002), habit formation (Knowlton *et al.*, 1996; Jog *et al.*, 1999), addiction (Gerdeman *et al.*, 2003; Everitt & Robbins, 2005) and other aspects of cognition (Middleton & Strick, 2000). The primary input nucleus of the BG is the striatum, which receives massive glutamatergic

input from all of the cerebral cortex (Kemp & Powell, 1970; McGeorge & Faull, 1989) and a number of thalamic nuclei (Beckstead, 1984; Berendse & Groenewegen, 1990; McFarland & Haber, 2000; Smith *et al.*, 2004). Both cortical and thalamic glutamatergic inputs synapse heavily onto the projection neurons of the striatum (Kemp & Powell, 1971; Zheng & Wilson, 2002), the medium spiny neurons (MSN).

The anatomic prominence of the corticostriatal (CS) afferent and the relative ease of preparing *in vitro* brain slices preserving this pathway have biased the study of excitatory synapses in the striatum to this afferent, despite the long-time knowledge of the existence of the thalamostriatal (TS) projections (Vogt & Vogt, 1941; Cowan & Powell, 1956). The thalamic nuclei participating in the TS projections are diverse in their cellular morphology (Deschenes *et al.*, 1995; 1996) and anatomic connections (Van der Werf *et al.*, 2002; Smith *et al.*, 2004; Fujiyama *et al.*, 2006; Raju *et al.*, 2006). Thalamic nuclei with heavy projections to the striatum have recently been shown to be involved in complex reward-related decision making (Kimura *et al.*, 2004; Minamimoto *et al.*, 2005), and these nuclei also degenerate in Parkinson's disease (Henderson *et al.*, 2000b; a). Because of the lack of information regarding this projection relative to the corticostriatal afferent pathway, we recently developed a novel rat brain slice preparation preserving the TS pathway in order to study the properties of this afferent pathway in more detail (Smeal *et al.*, 2007).

In the present paper, we test the hypothesis that synapse-specific differences in NMDA receptor content and properties exist at the TS and CS synapses onto MSNs, as synapse-specific differences have been discovered to be important in a number of brain regions (Toth & McBain, 1998; Weisskopf & LeDoux, 1999; Toth & McBain, 2000; Popescu *et al.*, 2007). The importance of NMDA receptor subunit composition, in particular the NR2 subunits, in the normal and pathological function of the basal ganglia has been demonstrated using a variety of experimental techniques including animal models of Parkinson's Disease (Blanchet *et al.*, 1999; Loschmann *et al.*, 2004), gene expression studies (Keefe & Adams, 1998; Keefe & Ganguly, 1998), and mathematical modeling studies (Wolf *et al.*, 2005). Additionally, NMDA/non-NMDA receptor ratios are thought to be critical for a number of brain functions (Lisman *et al.*, 1998; Perez-Otano & Ehlers, 2005; Popescu *et al.*, 2007). The present studies determine the NMDA receptor-mediated component of the post-synaptic currents in single MSNs resulting from stimulation of TS and CS afferent pathways. We found differences in both NMDA/non-NMDA receptor ratios and NR2B subunit content. These afferent pathway-specific differences could underlie fundamental functional differences between these two afferent pathways and also suggest that it may be possible to develop pharmacological approaches that target these different afferent pathways onto the MSNs for improved management of basal ganglia-related disorders.

Methods

Animals

Male Sprague-Dawley rats, ranging from 90 to 150 g, were used for the experiments. Rats were housed in groups in a room controlled for temperature and lighting on a 12-hr light/12-hr dark cycle. Rats had free access to food and water. All animal care and experimental manipulations were approved by the Institutional Animal Care and Use Committee of the University of Utah and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Striatal slice preparation

Acute brain slices were obtained as previously described (Smeal *et al.*, 2007). Rats were anesthetized with Nembutal (25 mg/kg) and decapitated. The brains were rapidly removed and

placed in ice-cold sucrose Ringer's solution bubbled with 95% O₂/5% CO₂ and containing (in mM): sucrose (124), KCl (3), NaPO₄ (1.2), MgSO₄ (2), NaHCO₃ (6), glucose (10), and CaCl₂ (2). The solution pH was between 7.34 and 7.36 and the osmolality was 295–305 mOsm. Previous tract tracing studies in brain slices indicated that a 30° off-horizontal, oblique, 400-μm thick rat brain slice preserved some of the parafascicular/centromedian (Pf/CM) thalamic pathway from the level of the reticular nucleus of the thalamus to the dorsal striatum (Smeal *et al.*, 2007). To achieve this cut, the brain was laid on a chilled cutting surface ventral side down and blocked along the midline. The cerebellum was removed, and the left hemisphere laid medial side down. The olfactory bulb was used as a reference to make the oblique 30° cut. The brain was bluntly blocked again rostrally and dorsally and then glued, ventral side down, on the Vibratome chuck. Slices were cut at a thickness of 400 μm while bubbling the slice in chilled sucrose Ringer's solution.

Whole cell patch clamp

Immediately after each slice was cut, it was placed in oxygenated normal Ringer's solution (NaCl substituted for sucrose) at room temperature and was incubated for one hr prior to experiments. For recording, slices were placed in a submersion chamber and constantly superfused at room temperature (1.5–2.0 ml/min) with oxygenated Ringer's solution. The whole-cell patch-clamp technique was used to record from spiny efferent neurons within striatum, as previously reported (Chapman *et al.*, 2003; Smeal *et al.*, 2007) using the blind technique (Blanton *et al.*, 1989). A microscope equipped with Nomarski optics and infrared illumination (Axioskop FS microscope, Zeiss) was used to visualize the slice, thus facilitating patching onto cells. Borosilicate patch electrodes (World Precision Instruments, FL) were pulled to 3–6 MΩ resistances using a micropipette electrode puller (Sutter Instruments, CA). Both potassium- and cesium-based internal solutions were used. The K-gluconate internal solution was comprised of (in mM): K gluconate (130), KCl (10), HEPES (10), EGTA-KOH (1), CaCl₂ (0.1), Na₂-ATP (4), Na-GTP (0.5) and glucose (10). The cesium-based internal solution was comprised of (in mM): CsCl (140 mM), HEPES (10), EGTA-CsOH (1), CaCl₂ (0.1), Na₂-ATP (4), Na-GTP (0.5), glucose (10), and QX-314 (10). Biocytin (1.5–2.5%) was added to the cesium internal solution. Cesium-containing electrodes increase membrane resistance and make the MSN more electrically compact (Spruston *et al.*, 1993). QX-314 (lidocaine N-ethyl bromide) blocks Na⁺ channels (Connors & Prince, 1982), GABA_B receptors (Lambert & Wilson, 1993), I_h channels (Perkins & Wong, 1995), and partially blocks some Ca²⁺ channels (Talbot & Sayer, 1996). When measured at a holding potential of −70 mV, the cesium-containing internal solution resulted in input resistances of 233 ± 5.4 MΩ (n = 13) compared to input resistances of 80.7 ± 3.5 MΩ (n = 10) for cells recorded with the potassium-based internal solution.

Concentric bipolar stimulating electrodes (Kopf Instruments, CA) were placed in the cortex adjacent to the corpus callosum for activation of the CS projection and in the reticular nucleus of the thalamus for stimulation of the TS projection, as previously described (Smeal *et al.*, 2007) (Figure 1). Stimulation was current controlled (100-μsec square pulses) and intensities just sufficient to evoke reliable and stable EPSCs were used. MSNs were selected from the dorsolateral striatum for whole-cell patch clamp recording (Figure 1). Picrotoxin (10 μM) was always included in the perfusion medium to block activation of GABA_A receptors. The Ringer's solution also included DL-2-amino-5-phosphonovaleric acid (APV; 100 μM) to block NMDA receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) to block AMPA/Kainate receptors, and ifenprodil (10 μM) and conantokin G (3 μM) to selectively block NR2B subunit-containing NMDA receptors as needed and described in the results section. All antagonists were purchased from Sigma except Conantokin G, which was a kind gift from the laboratory of Dr. B. Olivera. For maximal activation of all types of NMDA receptors, MgSO₄ was omitted from and 10 μM glycine was included in the Ringer's solution (Wilcox *et al.*, 1996).

Following electrophysiology experiments, the striatal slice was gently lifted from the recording chamber and placed in freshly prepared 4% paraformaldehyde for 24–48 hours at 4°C, followed by phosphate buffered saline (PBS) until processing (up to 1 week). To probe for biocytin, slices were rinsed twice in 1X PBS, and incubated in streptavidin, conjugated to Alexa 546 (Molecular Probes, CA; 1:500), at room temperature overnight. Slices were then incubated in DAPI for 30 minutes, followed by several PBS washes. Before cover slipping with ProLong Gold antifade reagent (Molecular Probes, CA), slices were placed in 80% glycerol/PBS until tissue became adequately clear (up to one hour).

Data acquisition and analysis

Whole-cell recordings were obtained from MSNs using a MultiClamp 700A amplifier (Molecular Devices, CA). Signals in current-clamp mode were acquired at 20 kHz and filtered at 10 kHz for off-line analysis using Clampfit 9 (Molecular Devices). Input and series resistance values of 50–120 MΩ and < 20 MΩ for recordings with the K⁺-based internal solution (100–350 MΩ and < 20 MΩ for cesium-based internal solution) were used as selection criteria for accepting recordings. Only recordings that did not exhibit substantial changes in resistance (<15%) were used for analysis. Cells with resting membrane potentials more depolarized than –60 mV were omitted from analysis. The following parameters were determined for averaged EPSCs in various experiments: NMDA/non-NMDA ratios, synaptic delays, peak amplitudes, decay time constants, and charge transfer. The decay times of the currents were fit with a double exponential of the form: $I(t) = I_f \cdot \exp(-t/\tau_f) + I_s \cdot \exp(-t/\tau_s)$, where I_f is the amplitude of the fast component, I_s is the amplitude of the slow component, and τ_f and τ_s are the fast and slow time constants, respectively. Weighted time constants were calculated using the equation: $\tau_w = [I_f / (I_f + I_s)] \times \tau_f + [I_s / (I_f + I_s)] \times \tau_s$ (Stocca & Vicini, 1998). All data are presented as mean ± SEM, unless otherwise noted. Because only MSNs that exhibited EPSCs in response to stimulation to both afferent pathways were used for analysis, the paired t-test was used for statistical comparisons. Decay kinetics for the TS- and CS-mediated EPSCs examined with the two different internal solutions were compared using a two-way, mixed factor ANOVA (afferent × internal solution) and the Bonferroni post-hoc test (Prizm, GraphPad Software, CA). Significance was set at $p < 0.05$.

Measurement of NMDA/non-NMDA ratios

NMDA/non-NMDA ratios were measured as area under the curve of the EPSC for the first 150 ms of the current measured from the stimulus artifact. Area under the curve of the EPSC is a measure of charge transfer and is not attenuated as strongly as peak current due to the electrotonic properties of dendritic arbors (Carnevale & Johnston, 1982; Major, 1993; Spruston *et al.*, 1993; Lisman *et al.*, 1998). However, because NMDA/non-NMDA ratios are often reported as the ratio of peaks, these ratios were calculated as well for comparison. Holding the membrane potential of the MSN at –70 mV, each pathway was alternately stimulated with a 2-s pause between stimulations and an 8-s pause between each recorded trace. After 10 min of baseline stimulation, drugs as described in the results section were washed onto the slice for 20 min. Averages of 25 traces were taken immediately before drug application and after 20 min of drug exposure.

Results

The glutamate receptor-mediated EPSCs examined in this paper were all collected from MSNs that demonstrated responses to stimulation of both TS and CS pathways. These cells represent approximately 50% of the MSNs that were patch clamped. When patch clamping with the potassium-based internal solution, MSNs were identified by their characteristic decreased input resistance (inward rectification) in response to hyperpolarizing current injection steps and the depolarization ramp in response to depolarizing current injection (Figure 1B). When using the

cesium-based internal solution containing QX-314, MSNs were identified by neuron filling with biocytin (Figure 1C). The synaptic delays measured from the stimulus artifact until the onset of the EPSC were 6.37 ± 0.37 ms for the TS pathway and 7.15 ± 0.56 ms for the CS pathway and were not significantly different overall (paired t-test; $p=0.127$, t ratio = 1.575, df = 27). Average amplitudes for the total EPSCs, NMDA receptor-mediated EPSCs, and AMPA/kainate receptor-mediated EPSCs were 65.4 ± 7.8 pA ($n=24$), 29.8 ± 3.7 pA ($n=27$), and 26.1 ± 7.2 pA ($n=11$) for the TS pathway and 81.2 ± 8.0 pA ($n=24$), 33.9 ± 5.2 pA ($n=27$), and 58.4 ± 12.4 pA ($n=11$) for the CS pathway, respectively.

NMDA/non-NMDA ratios at the TS synapse are greater than at the CS synapse

Examination of potential NMDA receptor differences between the TS and CS synapses started with the study of the contribution of the NMDA receptor-mediated current to the overall glutamate-mediated synaptic current, as measured by the NMDA/non-NMDA ratio. The NMDA/non-NMDA ratios were calculated by measuring the area under the total EPSC trace (including both NMDA and AMPA/kainate receptor-mediated currents), measuring the area under the trace remaining after APV application (AMPA/kainate receptor-mediated charge transfer), and subtracting the latter from the former to give the NMDA receptor-mediated portion. These areas were calculated for the first 150 ms of the EPSC trace, a time period encompassing the AMPA/Kainate receptor-mediated EPSC (Figure 2A, inset). This integration was calculated after subtracting the baseline measured immediately preceding the stimulation artifact and is a measure of total charge transfer in this interval of time. The result of these calculations are illustrated by the shaded regions of the insets in Figure 2A. All the ratios ($n=11$) are plotted in Figure 2B, and the pairing within each MSN is indicated by a connecting line. The data are presented in this way to emphasize that while there is variability of NMDA/non-NMDA ratios at the TS and CS synapses between cells, the relationship between the TS and CS synapses within a single neuron is consistent. An NMDA/non-NMDA ratio of 1, by this measure, means that the charge transferred in the first 150 ms is equal between NMDA and AMPA/Kainate receptors. A ratio greater than 1 indicates an EPSC dominated by NMDA receptor-mediated current. It can be seen that the TS synapse has a significantly greater NMDA/non-NMDA ratio (2.61 ± 0.67) than the CS synapse (1.52 ± 0.38 ; paired t-test, $p=0.0374$, t ratio = 2.398, df = 10, Figure 2C). NMDA/non-NMDA ratios are sometimes calculated in the literature using EPSC peak amplitudes (Watt *et al.*, 2000; Watt *et al.*, 2004; Popescu *et al.*, 2007). The lower bar graph in Figure 2C shows the NMDA/non-NMDA ratios calculated in this manner. In this case, there is a trend for the TS ratio to be greater, although the difference between TS (1.68 ± 0.72) and CS synapses (0.83 ± 0.36) did not reach statistical significance (paired t-test; $p=0.0959$, t ratio = 1.838, df = 10).

A second set of experiments was done to calculate NMDA/non-NMDA ratios using a cesium-based internal solution for the patch pipette and blocking AMPA/Kainate receptors using CNQX (10 μ M). Similar to the approach described above, after 10 min of baseline stimulation, CNQX was applied for 20 min and the remaining NMDA receptor-mediated EPSC measured (Figure 2D). NMDA/non-NMDA ratios were calculated using the method discussed earlier except that in this case the NMDA receptor-mediated current is measured directly and the AMPA/Kainate receptor-mediated current calculated via a subtraction. All the ratios are plotted in Figure 2E with connecting lines linking the TS and CS NMDA/non-NMDA ratios measured in the same MSN ($n=12$). Again, the TS-mediated EPSCs had significantly higher NMDA/non-NMDA ratios (2.61 ± 1.1) when compared to the CS-mediated EPSCs (0.99 ± 0.41 ; paired t-test; $p=0.0364$, t ratio = 2.383, df = 11). A bar graph showing the ratios calculated using peaks (TS 1.37 ± 0.27 , CS 0.74 ± 0.25) is shown in Figure 2F. This measure also revealed significantly higher NMDA/non-NMDA ratios in the TS pathway (paired t-test; $p=0.002$, t ratio = 3.97, df = 11). In comparing both APV and CNQX experiments, a two-way analysis of variance revealed that there was no main effect for the antagonist used to determine the ratios

(APV vs. CNQX; $F_{1,20}=0.024$, $p=0.878$) and no significant interaction between drug and afferent pathway ($F_{20,20}=4.74$, $p=0.471$), providing assurance in the validity of the NMDA/non-NMDA ratios. There was, however, a significant main effect for afferent pathway, with the NMDA-nonNMDA ratios significantly greater for the thalamostriatal synapse ($F_{1,20}=10.69$, $p=0.004$). Two cells in the APV experiments (Figure 2B) and one cell in the CNQX experiments (Figure 2E) exhibited relatively high NMDA/non-NMDA ratios implying large proportions of NMDA receptors at these synapses. Using the Grubb's test for statistical outliers, neither of the two cells shown in Figure 2B are statistical outliers. The cell in Figure 2E is a statistical outlier using this test ($Z = 3.06$, $p<0.0001$). However, exclusion of this cell does not alter the significance (paired t-test, $p_{\text{new}}=0.002$ vs. $p_{\text{old}} = 0.036$). Because these potential outlier cells occur with a similar frequency across different experimental protocols, we feel they most likely represent real physiological differences and have therefore chosen to retain them all in the results; and the data are shown in their entirety in Figure 2.

Reversal potentials suggest that TS synapses are more electrotonically distant

Thalamic input from midline thalamic nuclei is biased to more distal locations of the dendritic arbor of CA1 hippocampal pyramidal neurons (Wouterlood *et al.*, 1990; Megias *et al.*, 2001). This more electrotonically distant location is also associated with higher NMDA/non-NMDA ratios and reversal potentials that deviate from the theoretical due to space clamp errors (Otmakhova *et al.*, 2002). To test if an analogous situation exists in the striatum, reversal potential experiments were performed. A more positive reversal potential for an EPSC is suggestive of a more distant location of the synapses on the dendritic arbor due to the cable properties of these processes (Carnevale & Johnston, 1982; Major, 1993; Spruston *et al.*, 1993). For these experiments, cesium and QX-314 were included in the patch pipette solution, blocking potassium and sodium channels, respectively. Additionally, QX-314 is known to partially block some Ca^{2+} channels (Talbot & Sayer, 1996). Pipette junction potentials were measured and corrected for using a Multiclamp 700A amplifier. To calculate reversal potentials, MSNs were voltage clamped to holding potentials ranging from -70 to $+70$ mV in incremental steps of 10 mV. At each potential, 10-15 traces were collected for averaging, corresponding to 2-3 minutes of recording at each potential. Voltage-activated Ca^{2+} channels not blocked by QX-314 were observed, but these inactivated within a few hundreds of ms after stepping the holding voltage and did not interfere with the EPSCs evoked from TS and CS pathway stimulation. Examples of average traces from a subset of the holding potentials for one experiment are shown in Figure 3A. The peak amplitudes of the TS- and CS-mediated EPSCs were calculated at each potential and plotted, and the EPSC peak amplitudes vs holding voltage was plotted and fit with a line to determine reversal potential for each MSN. The average normalized EPSC for each holding potential is shown for the CS and the TS synapses in Figure 3B. The reversal potentials for TS- and CS-mediate EPSCs for all MSNs tested are shown in Figure 3C. As with Figure 2B and 2E, the reversal potentials for the TS and CS synapse from the same MSN are connected with a line. The reversal potentials at the TS and CS synapses showed variability between cells, however the relative relationship within each MSN was consistent. The average reversal potentials for cortical (14.9 ± 1.6 mV) and thalamic (19.1 ± 1.9 mV) stimulation are shown in Figure 3D and were found to be significantly different (paired t-test; $p = 0.0006$, t ratio = 5.179, $df = 9$). The more positive reversal potential seen for the thalamic synapse is further from the theoretical reversal potential of zero for these receptors, suggesting a larger space-clamp error and thus a more electrotonically remote synapse (Carnevale & Johnston, 1982; Major, 1993; Spruston *et al.*, 1993).

Conantokin G inhibits NMDA receptor-mediated EPSCs to a greater extent at the CS synapse

Synapse-specific targeting of particular NMDA receptor subunits has been observed in a number of brain regions, and these differences have been hypothesized to contribute to differential responses of the postsynaptic cell to each distinct afferent pathway (Weisskopf &

LeDoux, 1999; Kumar & Huguenard, 2003). To test for subunit differences in NMDA receptors mediating TS vs. CS neurotransmission, NMDA receptor antagonists specific for NR2B-containing receptors were used. NMDA receptor-mediated EPSCs were isolated with picrotoxin (50 μ M) and CNQX (10 μ M). After 10 min of baseline stimulation, ifenprodil (10 μ M) or Conantokin G (3 μ M) were washed on for 20 min. Figure 4A shows examples of average traces before and after the application of ifenprodil. The matched data points representing the percentage of EPSC peak reduction following drug application are shown in Figure 4B. There was no significant difference between the TS and CS pathways in response to ifenprodil treatment (paired t-test, $p = 0.52$, t ratio = 0.70, $df = 5$). A two-way, mixed-factor ANOVA examining NMDA receptor-mediated EPSC amplitudes (Ifenprodil $\times$ afferent), revealed no overall main effects for either afferent pathway stimulated ($F_{1,10}=0.90$, $p=0.37$) or drug application ($F_{1,10}=0.02$, $p=0.88$) and no significant interaction ($F_{10,10}=6.13$, $p=0.96$, Figure 4C). Past observations from our laboratory showed a large reduction of NMDA receptor-mediated EPSCs by ifenprodil in young rats and a smaller but significant reduction of NMDA receptor-mediated EPSCs in older rats (Chapman *et al.*, 2003). In the previous paper, however, EPSCs were elicited by local stimulation within the striatum, while in the present paper we are specifically activating the CS or TS afferent pathways. It is known that triheteromeric NMDA receptors are more prevalent at the synapse (Dunah & Standaert, 2003). The local stimulation utilized previously most likely activated both synaptic and extrasynaptic NMDA receptors, which would include more diheteromeric, NR1/NR2B NMDA receptors that are more susceptible to block by ifenprodil. In the present experiments, we are specifically activating NMDA receptors at the synapse, which contain more triheteromeric NMDA receptors and would show less sensitivity to ifenprodil.

The second antagonist used, conantokin G, is a toxin that has been shown to have specificity for NR2B-containing NMDA receptors (Donevan & McCabe, 2000). Example average EPSC traces before and after application of Conantokin G are shown in Figure 4D. Conantokin G produced a greater reduction of the EPSCs elicited by CS stimulation compared to the EPSCs elicited by stimulation of the TS pathway (paired t-test; $p = 0.0008$, t ratio = 6.17, $df = 6$). The effects of conantokin G on EPSCs mediated by the different afferent pathways are shown in Figure 4E and are consistent with greater NR2B content in NMDA receptors mediating CS vs. TS transmission (Barton *et al.*, 2004; Alex *et al.*, 2006). A two-way, mixed-factor ANOVA examining NMDA receptor-mediated EPSC amplitudes (conantokin G $\times$ afferent) was done to examine the effect of conantokin G on NMDA receptor-mediated EPSC amplitude in more detail (Figure 4F). This analysis revealed a significant interaction between conantokin G application and afferent pathway ($F_{12,12}=34.96$; $p = 0.001$). Additionally, there were overall main effects for both conantokin G application ($F_{1,12}=58.47$, $p<0.0001$) and afferent pathway stimulated ($F_{1,12}=4.76$, $p=0.049$). In the conantokin G experiments, the control EPSCs elicited from CS pathway stimulation were generally larger than the EPSCs elicited from TS pathway stimulation (Figure 4F). In order to test the possibility that more NR2B-containing receptors were recruited at higher EPSC magnitudes, a Pearson correlation test was run between the cortical EPSC amplitudes (range: 32.2 pA to 121 pA) and the corresponding percent block by conantokin G. This test resulted in a Pearson $r = -0.75$ ($r^2 = 0.56$) which was not statistically significant. These results suggest that while there is greater NR2B content in the NMDA receptors of the CS pathway, some NR2B-containing receptors are also present at the TS synapse.

EPSC decay kinetics at TS and CS pathway synapses are different

The incorporation of the NR2A subunit into the NMDA receptor is known to contribute to faster decay kinetics for NMDA receptor-mediated EPSCs (Kirson & Yaari, 1996; Flint *et al.*, 1997; Vicini *et al.*, 1998). If the CS synapses do utilize a higher proportion of NR2B-containing NMDA receptors as the pharmacology suggests, then the decay kinetics of the EPSC

should be slower. The decay kinetics of MSN EPSCs elicited from both CS and TS pathway stimulation were measured using both cesium-based and potassium-based internal solutions. The cesium-based internal solution increases the membrane resistance and minimizes the distortion of EPSCs arising from the electrotonic properties of dendrites. The potassium-based internal solution, which resembles a more physiological state, causes a lower membrane resistance and should cause changes in the measured kinetic properties compared with the cesium internal if the TS synapses are consistently more distant than the CS synapses, as suggested by the reversal potential experiments. Fitting the NMDA receptor-mediated EPSC decay with a double exponential produces slow and fast time constants and slow and fast amplitudes, one for each exponential term (Figure 5A, inset). Table 1 lists the slow and fast time constants (msec), the weighted time constant (msec), and the percent of the total amplitude represented by the fast time constant. For each variable, a two-way, mixed-factor ANOVA (internal solution $\times$ afferent) was done. This analysis revealed a significant interaction between internal solutions for the slow time constant ($F_{30,30}=2.02$, $p < 0.0001$) and the weighted constant ($F_{30,30}=1.48$, $p = 0.027$), and a trend for a significant interaction for the fast time constant ($F_{30,30}=0.78$, $p = 0.08$). A Bonferroni post hoc test showed that in the cesium condition, the slow time constant was significantly slower for the NMDA receptor-mediated EPSCs evoked by stimulation of the CS pathway compared to those evoked by stimulation of the TS pathway ($p < 0.05$, $t = 2.51$), consistent with the hypothesis that there is greater NR2B content in the CS pathway. For the EPSCs measured with the potassium-based internal solution, post hoc tests showed significantly slower slow and weighted time constants for the TS pathway ($p < 0.05$, $t = 2.58$), consistent with data from the reversal potential experiments implying that the TS pathway is more electrotonically distant. These observations are summarized in Figure 5.

Discussion

The CS and TS afferent pathways are the prominent excitatory pathways to the striatum. Anatomic and behavioral studies suggest fundamentally different functions for these two pathways, but direct comparisons of the characteristics of these afferent pathways have been lacking. Utilizing a novel rat brain slice preparation, we have identified several fundamental differences in excitatory synaptic transmission in MSNs that are innervated by these two distinct pathways. We describe here significant differences in NMDA/non-NMDA ratios, decay kinetics of the NMDA receptor-mediated EPSC, and NMDA receptor pharmacology between the TS and CS pathways in the same neurons, which strongly suggest afferent-selective differences in synaptic function.

We have found that the synapses mediating TS input to a given MSN have a greater NMDA/non-NMDA ratio than do the synapses mediating CS input to the same MSN. This relationship was the same regardless of whether the ratio was calculated by blocking NMDA or AMPA/Kainate receptors or whether cesium or K-gluconate was used in the internal solution. The NMDA/non-NMDA ratios calculated using areas, as opposed to peak EPSC values, appear more sensitive to detecting ratio differences; this is congruent with theoretical work suggesting that charge transfer is a better measure in neurons where efficacious space-clamp is difficult (Carnevale & Johnston, 1982; Major, 1993; Spruston *et al.*, 1993). The relationship observed in the present study is opposite to recently published results examining CS and TS synaptic differences in our thalamostriatal slice preparation adapted to the mouse (Ding *et al.*, 2008). The basis for this difference currently is not clear, but could be related to differences in the animal model (rat vs. mouse), recording electrode position in the striatum (our position is more rostral and dorsal), and age of the animals (our rats are 28-42 days vs. 21-31 days for the mouse study). NMDA receptor content in the striatum is known to vary during this developmental time period and also varies positionally across the striatum (Watanabe *et al.*, 1993; Chapman *et al.*, 2003). Additionally, different thalamic nuclei projecting to striatum have different

anatomic and electrical characteristics (Deschenes *et al.*, 1995; Raju *et al.*, 2006; Lacey *et al.*, 2007). Clearly, future studies will need to directly compare these parameters to assess their impact on excitatory transmission in the CS and TS pathways.

Comprehensive mathematical models of MSNs suggest that NMDA/non-NMDA ratios may be important in regulating the synchronization frequencies of the MSN with its afferent pathways (Wolf *et al.*, 2005). Higher NMDA/non-NMDA ratios have been hypothesized to allow for self-regenerating dendritic events similar to the action potential (Schiller & Schiller, 2001) or to potentially function as an ideal current source (Cook & Johnston, 1999). Therefore, NMDA/non-NMDA ratios have the potential to powerfully modulate the functionality of different types of synapses. The length of the decay of NMDA receptor-mediated EPSCs can be modulated by both NMDA/non-NMDA ratios and receptor subunit content, and afferent synapse-specific regulation of these parameters could therefore set synchronization frequencies of MSNs with these two pathways. Likewise, changes in subunit expression, as observed in Parkinson's disease (Dunah *et al.*, 2000; Hallett *et al.*, 2005), could contribute to pathological synchronization frequencies between the striatum and its afferent brain regions.

Reversal potential experiments presented here suggest that both TS and CS synapses are electrotonically distant on the MSN dendritic arbor, with the TS pathway forming synapses at more distant locations. A more distant location on the dendritic arbor for the TS synapse is implied by the more positive reversal potential for the TS pathway and is consistent with the observed sensitivity of the NMDA receptor-mediated EPSC decay kinetics to membrane resistance manipulations (Figure 5). It has been difficult using anatomical techniques to detect any biases of striatal afferent pathways to specific portions of the dendritic arbors of MSNs due to the lack of distinct layers in the striatum. However, the magnitude of the differences between the TS and CS reversal potentials is similar to those observed for excitatory synapses onto the different dendritic layers of pyramidal neurons of the hippocampus (Otmakhova *et al.*, 2002). Interestingly, our data showing that the more distant TS synapses also exhibit higher NMDA/non-NMDA ratios are analogous to the synapses made by projections of the thalamic reuniens nucleus to the stratum lacunosum-moleculare of CA1 hippocampus (Wouterlood *et al.*, 1990; Otmakhova *et al.*, 2002). The analogy continues with the observations that both the Pf thalamic projection to striatum (Sadikot *et al.*, 1992; Raju *et al.*, 2006) and the reuniens thalamic projection to hippocampus (Megias *et al.*, 2001) are biased to dendritic shafts. These similarities raise the possibility that such properties are conserved among the projections of the “non-specific nuclei” of the thalamus and that these nuclei may thus generally exert potent influence on neuronal function.

In the present work, the reversal potentials of both the CS and TS afferents differ significantly from the theoretical reversal potentials for ionotropic glutamate receptors. These differences are expected given the nature of the MSN dendritic arbor and the resulting space-clamp errors (Spruston *et al.*, 1993). Spruston *et al.* have shown in their model that although cesium-based internal solutions significantly reduce space clamp errors, they do not eliminate them. Recently, there has been experimental verification of the space clamp errors predicted by the Spruston *et al.* model, demonstrating large errors in the measurement of reversal potentials at distal dendritic locations even under optimal recording conditions (Williams & Mitchell, 2008). While space clamp errors can complicate the interpretation of recordings made at the soma, the accurate description of these errors (Spruston *et al.*, 1993; Williams & Mitchell, 2008) allows us to test the relative position of synapses on the dendritic arbor as variation in access and membrane resistance should preserve the relative relationship of the reversal potentials at different synaptic locations within a single neuron. However, active (nonlinear) voltage-dependent channels could confound the interpretations, as the cesium and QX-314 used in the present study do not block all voltage-dependent calcium channels. These currents inactivated on the order of 100s of ms and so we do not think they played a role in determining the

differences in reversal potentials that we observed; however, we cannot fully exclude a role of differences in calcium loading in the neuron.

The pharmacological studies reported herein suggest that NMDA receptors mediating CS input have a greater contribution of the NR2B subunit. Although ifenprodil has been shown to be a specific antagonist for NR2B-containing receptors (Williams, 1993; 2001), it has less effect when receptors also contain an NR2A subunit (i.e. are triheteromeric receptors) (Hatton & Paoletti, 2005). Such reduced efficacy at NR2A-containing receptors is also suggested by striatal slice experiments in which suppression of NMDA receptor-mediated EPSCs by ifenprodil is large in young rats but smaller in older rats when more NR2A subunits are present (Chapman *et al.*, 2003). The lack of an effect of ifenprodil on either CS- or TS- mediated NMDA-receptor mediated EPSCs suggests that the majority of synaptic NMDA receptors in the striatum of adolescent rats contain at least one NR2A subunit and are triheteromeric, as has been suggested by other groups (Dunah & Standaert, 2003). The lack of any suppression of CS- or TS- mediated NMDA-receptor mediated EPSCs in the present slice preparation differs from the significant reduction seen following local stimulation in previous work (Chapman *et al.*, 2003). In the present work, TS and CS synapses are specifically activated by appropriate placement of the stimulating electrodes, thus preferentially activating synaptic triheteromeric receptors. In the previous studies, local stimulation likely activated both synaptic triheteromeric NMDA receptors and extra-synaptic diheteromeric NMDA receptors, the latter of which are more susceptible to block with ifenprodil.

Conantokin G has been observed to inhibit NMDA receptor-mediated EPSCs in cultured cortical neurons and in rat CA1 brain slices (Barton *et al.*, 2004; Alex *et al.*, 2006), suggesting that this drug has better efficacy than ifenprodil against triheteromeric NMDA receptors. In the present studies, conantokin G produced significantly greater inhibition of NMDA receptor-mediated EPSCs evoked by CS vs. TS stimulation. These findings suggest that the CS synapses may contain more triheteromeric NMDA receptors whereas the TS synapses may contain NR1/2A diheteromeric receptors (i.e. Conantokin G insensitive) as well as triheteromeric receptors. The slower EPSC decay kinetics observed for the CS afferent pathway when recordings were done with the cesium-based internal solution are consistent with the conclusion that there is more NR2B incorporation into NMDA receptors at these synapses. As with the NMDA/non-NMDA ratios, our observation of relatively higher NR2B content at the CS synapse is opposite that recently reported for a similar slice preparation from the mouse (Ding *et al.*, 2008). Again, this difference could be due to the animal model, differences in developmental time point, or position of recording in the slice preparation, and the basis for this difference will require additional studies systematically manipulating these variables.

The importance of NMDA receptor subunit distribution, in particular the NR2 subunits, in the normal and pathological function of the basal ganglia has been demonstrated using a variety of experimental techniques including animal models of Parkinson's disease (Hallett & Standaert, 2004; Fan & Raymond, 2007), gene expression studies (Keefe & Adams, 1998; Keefe & Ganguly, 1998), and mathematical modeling studies (Wolf *et al.*, 2005). The distribution and subunit composition of the NMDA glutamate receptor has also recently received considerable attention in the context of normal basal ganglia function and the pathophysiological function leading to the symptoms of Parkinson's disease (Hallett & Standaert, 2004). In animal models of Parkinson's disease, the ameliorative effect of dopamine replacement is augmented by NMDA receptor antagonists (Papa & Chase, 1996; Blanchet *et al.*, 1999). Additionally, NMDA receptor antagonists may reduce L-DOPA-induced dyskinesia (Blanchet *et al.*, 1999; Loschmann *et al.*, 2004). The differences between NMDA receptor expression at the CS and TS synapses onto MSNs observed in the present study provide a potential anatomic substrate for the observed actions of various NMDA receptor subunit-specific antagonists, and the therapeutic promise of NMDA receptor subunit-specific

antagonists as an adjunct therapy for the treatment of Parkinson's disease may be due to the ability to target one afferent pathway over the other. The present observations are consistent with recent work that has shown differential reorganization of TS and CS glutamate synapses in a monkey model of Parkinson's disease (Raju *et al.*, 2008) and suggest that alterations in the glutamate system following dopamine loss contribute to the symptoms of Parkinson's disease. Synapse specific subunit changes may be of importance in the symptoms and progression of Huntington's disease as well. Alterations in the CS pathway appear to be important in the progression of the disease (Cepeda *et al.*, 2003) and CS pathway degeneration may be protective against MSN death (McGeer *et al.*, 1978; Cepeda *et al.*, 2003). The pathology of the disease is also associated with the enhancement of NR2B containing receptors (Chen *et al.*, 1999; Zeron *et al.*, 2002; Li *et al.*, 2004). The present observation of higher NR2B content at the CS synapse is consistent with these observations.

The dominant component of the TS projection arises from the Pf/CM nucleus and projects to the dorsolateral striatum. It is interesting not only because of its size, but also its distinctive bias to dendritic shafts (Herkenham & Pert, 1981; Fujiyama *et al.*, 2006; Raju *et al.*, 2006), its involvement in Parkinson's Disease (Henderson *et al.*, 2000b; a), and its modulation of arousal and cognitive state (Baars, 1995; Smythies, 1997; Matsumoto *et al.*, 2001; Minamimoto & Kimura, 2002; Van der Werf *et al.*, 2002). Due to the increasing interest in this prominent nucleus of the TS projection, we developed this rat brain slice preparation to preserve this afferent as best as possible and determined stimulation locations that should bias activation to the Pf/CM component in order to study these synapses (Smeal *et al.*, 2007). The choice of slice angle and stimulation location was based on Pf/CM tract tracing studies in the slice (Smeal *et al.*, 2007) and the anatomical observation that the intralaminar nuclei of the thalamus are biased to the rostral portion of the reticular nucleus of the thalamus (Kolmac & Mitrofanis, 1997; Deschenes *et al.*, 1998). Despite these attempts, the actual source of the thalamic afferents stimulated in the current experiments is not known. Clearly future refinements allowing for specific activation of distinct TS afferents are necessary and important to determine the extent to which the general features of the synapses mediating TS input identified in the present work are common for all TS afferent pathways or specific, for example, to those arising from the Pf/CM complex. This refinement is all the more important in light of the significant differences between our results and those recently published by Ding *et al.* (Smeal *et al.*, 2007; Ding *et al.*, 2008). An additional caveat is the potential activation of corticofugal fibers with collaterals to the striatum. Previous collision experiments testing for unwanted antidromic activation of these fibers suggested this was not a problem, but the existence of these fibers should be kept in mind in experiments involving the activation of multiple pathways from distant locations in a slice preparation.

The nuclei of the thalamus and their interaction with the cortex and BG are crucial for generating the oscillations correlated with different brain states (Steriade *et al.*, 1993; Steriade, 2000; Murer *et al.*, 2002). *In vivo* extracellular recordings in behaving rats have demonstrated that different regions of striatum synchronize at specific frequencies with the afferent brain regions innervating those striatal regions and that this synchronization correlates with specific behaviors (Berke *et al.*, 2004). NMDA/non-NMDA ratios may be important in regulating these synchronization frequencies (Wolf *et al.*, 2005). These theoretical predictions and experimental observations suggest that pathological changes in NMDA/non-NMDA ratios at various synapses in response to the dopamine denervation of PD may disrupt normal plasticity and synchronization of the striatum with its afferents and could contribute to the symptoms of the disease (Hallett & Standaert, 2004). These ratios could be altered through modulation of NMDA receptor number and/or subunit composition. Given the dominant role of the thalamus and cortex in orchestrating oscillations in relation to brain states (Steriade *et al.*, 1993; Steriade, 2000), it is critical to understand NMDA receptor subunit composition at the TS and CS synapses, as these may constrain interaction between the basal ganglia and these afferent

structures in the normal and diseased brain. The present study demonstrates that the TS and CS synapses utilize different NMDA/non-NMDA ratios and NMDA receptor subunits providing a physiological substrate that could mediate afferent pathway-specific modulation in the striatum.

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Abbreviations

BG, basal ganglia; CS, corticostriatal; MSN, medium spiny neuron; Pf/CM, parafascicular/centromedian; TS, thalamostriatal.

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Figure 1.

The rat brain slice preparation and identification of MSNs. A, Image of specific slice preparation with position of the recording electrode in the dorsolateral striatum (lines) and stimulating electrodes (circles) shown. Scale bar: 2000 μm . B, Example of voltage response of a MSN to a series of current injections. MSNs exhibit an inward rectification in response to hyperpolarizing current injections. Depolarizing current injections result in depolarization ramps before the action potential. C, Images of a MSN filled with biocytin during recording. Scale bar: 50 μm . The inset image demonstrates the spines of the MSN. Scale bar: 10 μm .

Figure 2.

NMDA/non-NMDA ratios are significantly greater at TS synapses compared to CS synapses. A, Traces of EPSCs recorded in a single MSN elicited by thalamic (left) and then cortical (right) stimulation, 2 s apart. The control EPSC and the residual EPSC following 20 min application of APV (100 μ M) to block the NMDA component are shown. NMDA/non-NMDA ratios are calculated using the area under the first 150 ms of the EPSC following the stimulus artifact. The NMDA receptor-mediated charge transfer is calculated by subtracting the area of the AMPA/Kainate receptor mediated EPSC from the control. These calculated areas are graphically shown in the inset. B, Plot of all the NMDA/non-NMDA ratios calculated for both TS and CS EPSCs. Lines connect matching data points for each cell. C, The top bar graph summarizes data demonstrating that the average TS NMDA/non-NMDA ratios are greater than the CS ratios. The lower bar graph shows the NMDA/non-NMDA ratios calculated similarly, but using peak EPSC amplitudes. D, Traces illustrating EPSCs elicited from thalamic (left) and then cortical (right) stimulation. Control EPSCs and residual EPSCs after 20-min application of CNQX (10 μ M) to block the AMPA/Kainate receptor component of the EPSCs are shown. NMDA/non-NMDA ratios are calculated using the area under first 150 ms of the EPSC following the stimulus artifact. The AMPA/Kainate receptor-mediated charge transfer is calculated by subtracting the area under the NMDA receptor-mediated EPSC from the control EPSC. E, Plot of all the NMDA/non-NMDA ratios calculated for both TS and CS EPSCs. Lines connect matching data points for each cell. F, The top bar graph shows the average for TS and CS NMDA/non-NMDA ratios. The lower bar graph shows the NMDA/non-NMDA ratios calculated similarly, but using peak EPSC amplitudes.

Figure 3.

Reversal potentials for the AMPA/NMDA EPSCs at TS synapses are more depolarized than those of CS synapses indicating a more distant location on the dendritic arbor. **A**, Sample average EPSCs (10 traces each) at various holding potentials. Stimulation artifacts have been removed for clarity. **B**, Averages of normalized EPSC amplitudes at each holding potential for CS-evoked EPSCs (upper) and TS-evoked EPSCs (lower; $n = 10$). **C**, Summary of all the reversal potentials for TS- and CS-evoked EPSCs. Matching data points within each cell are connected with a line. **D**, Bar graph of the average reversal potential as calculated with a linear fit for each experiment.

Figure 4.

Ifenprodil (10 μ M) and Conantokin G (3 μ M) differentially affect TS- and CS-evoked NMDA receptor-mediated EPSCs. A, Example traces of NMDA receptor-mediated EPSCs evoked by TS or CS stimulation before (black trace) and after (gray trace) application of ifenprodil (10 μ M). B, Plot of the peak EPSC (as percent of control) after ifenprodil application for individual neurons. C, Bar graphs showing average EPSC peaks for both TS and CS synapses before and after drug application. D, Example traces of TS and CS NMDA receptor-mediated EPSCs before (black trace) and after (gray trace) application of Conantokin G. E, Plot of the peak EPSC remaining (as percent of control) after application of conantokin G for individual neurons. F, Bar graphs showing average EPSC peaks for both TS and CS synapses before and after drug application. There was an overall effect for both drug application (*) and afferent pathway (Conantokin G application, $p < 0.0001$; afferent pathway, $p = 0.049$). Post hoc analysis revealed a significant conantokin G-mediated-reduction for the CS pathway (**), as well as a significant interaction between the two variables in peak EPSC amplitude ($p = 0.001$).

Figure 5.

Bar graphs showing the different decay kinetics for TS and CS NMDA receptor-mediated EPSCs using cesium-based internal solution (top graph, higher membrane resistance) and potassium gluconate-based internal solution (lower graph, lower membrane resistance). A, Bar graphs displaying fast, slow and weighted time constants for the EPSCs evoked via CS and TS pathway stimulation in MSNs with cesium in the pipette solution. B, Bar graphs displaying fast, slow and weighted time constants for the EPSCs evoked via CS and TS pathway stimulation in MSNs with potassium gluconate in the pipette solution. The slow and weighted time constants were significantly slower in the TS pathway (Bonferroni posttest, $n = 16$, $p < 0.01$). There was a significant interaction due to internal solution for slow and weighted time constants (2-way ANOVA, $p < 0.0001$).

Kinetic properties of NMDAR-EPSCs in thalamostriatal and corticostriatal afferents
Summary table showing average values derived from two-component exponential fits to the decay phase of NMDA receptor-mediated EPSCs.

	Striatal Afferent	τ fast	τ slow **	% Fast	τ weighted **
Cesium (n=16)	Thalamostriatal	77.4 $\pm$ 9.8	409 $\pm$ 48.9 *	72.6 $\pm$ 6.7	193 $\pm$ 37.2
	Corticostriatal	105 $\pm$ 12.1	661 $\pm$ 75.1	77.8 $\pm$ 3.1	233 $\pm$ 25.3
K-Gluconate (n=16)	Thalamostriatal	62.5 $\pm$ 4.1	719 $\pm$ 118 *	69.2 $\pm$ 4.0	304 $\pm$ 75.9 *
	Corticostriatal	55.9 $\pm$ 8.0	366 $\pm$ 54.7	66.1 $\pm$ 5.0	155 $\pm$ 22.6

* The slow time constant was significantly slower in the CS pathway using the *cesium-based internal* (Bonferroni posttest, n= 16, p < 0.05). Both slow and weighted time constants were significantly slower in the TS pathway using the *K-gluconate-based internal* (Bonferroni posttest, n = 16, p < 0.01).

** There are significant interactions due to internal solution for slow and weighted time constants (2-way ANOVA, p<0.0001).