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Group III Metabotropic Glutamate-Receptor Mediated Modulation of Excitatory Transmission in Rodent Substantia Nigra Pars Compacta Dopamine Neurons

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Running Title: Group III mGLuRs modulate excitatory transmission in SNc

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Nonstandard abbreviations: (2S)-3-[[(1S)-1-(3,4-chlorophenyl)ethyl]amino-2-hydroxypropyl]phosphinic acid (CGP55845), R,S)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), (S)-3,4-Dicarboxyphenylglycine ((S)-3,4-DCPG), L-(+)-2-Amino-4-phosphonobutyric acid, (L-AP4), metabotropic glutamate receptor (mGluR), Parkinson's disease (PD), N-Phenyl-7-(hydroxymino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), substantia nigra pars compacta (SNc).

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Abstract

Glutamate plays an important role in the regulation of dopamine neuron activity. In particular, the glutamatergic input from the subthalamic nucleus is thought to provide control over dopamine neuron firing patterns. The degeneration of dopamine neurons in the substantia nigra pars compacta (SNc) observed in Parkinson's disease (PD) is believed to be due to a complex interplay of factors including oxidative stress and mitochondrial dysfunction. While glutamate is not the primary cause of cell death in PD, there is evidence suggesting excessive glutamate release onto dopamine neurons may play a role in continued degeneration. While many studies have focused on the role of glutamate in the SNc, little work has been directed at exploring the modulatory control of glutamate release in this region. Previous studies have found a high potency inhibitory effect of non-selective group III mGluR agonist on glutamatergic transmission in the SNc. Using whole cell patch clamp methods and novel pharmacological tools we have determined that mGluR4 mediates the group III-mGluR modulation of excitatory transmission in the rat SNc. The Group III mGluR-selective agonist L-(+)-2-amino–4-phosphonobutyric acid inhibits excitatory transmission in the SNc at low micromalar concentrations with a maximal inhibition occurring at 3μM. This effect was potentiated by the mGluR4-selective allosteric modulator N-Phenyl-7-(hydroxymino)cyclopropa[b]chromen-1a-carboxamide, and was not mimicked by the mGluR8-selective agonist (S)-3,4-Dicarboxyphenylglycine. Interestingly, in an attempt to employ knockout mice to confirm the role of mGluR4, we discovered an apparent species difference suggesting that in mice, both mGluR4 and mGluR8 modulate excitatory transmission in the SNc.

SNc neurons recorded in vivo exhibit both pacemaker-like and burst firing patterns (Grace and Bunney, 1984a;Grace and Bunney, 1984b;Tepper et al., 1995). The finding that SNc neurons recorded in brain slice preparations only exhibit pacemaker like activity has highlighted the importance of afferent inputs in determining the firing pattern of these cells. Anatomical and electrophysiological evidence exists for a direct excitatory projection from the subthalamic nucleus (STN) to the dopaminergic neurons in the SNc (Smith et al., 1990;Smith and Grace, 1992;Iribe et al., 1999) that may in part modulate SNc bursting (Smith and Grace, 1992). This glutamatergic input has been the focus of much attention based on the findings that the STN is overactive in Parkinson's disease (PD) and the fact that glutamate is believed to play a role in the continued degeneration of the SNc dopamine neurons in this disorder (Rodriguez et al., 1998). However, relatively little work has been focused on the modulatory control of these excitatory inputs.

The metabotropic glutamate receptors (mGluRs) are a class of G-protein coupled receptors that play a variety of neuromodulatory roles in the central nervous system. To date, eight mGluR subtypes have been cloned (for review see (Conn and Pin, 1997)). The mGluRs are classified into 3 groups based on sequence homology, agonist and antagonist pharmacology, and coupling to signal transduction pathways in expression systems. Group I (mGluR1 and mGluR5) mGluRs are preferentially expressed in postsynaptic region were they modulate cell excitability. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs are presynaptically localized and modulate the release of neurotransmitter. Based on the localization in the basal ganglia motor loop, and the functional roles that have been

revealed to date, the group III mGluRs, and mGluR4 in particular hold promise for the design of novel palliative treatments for PD (Marino et al., 2003a).

We previously have shown that presynaptically localized mGluR4 modulates inhibitory transmission at the striatopallidal synapse, a critical synapse in the indirect pathway of the BG motor system (Valenti et al., 2003). Furthermore, when studied in both acute and chronic behavioral models of PD, activation of mGluR4 produces a dramatic reversal of motor impairment suggesting mGluR4 may provide a potential target for the palliative treatment of PD (Valenti et al., 2003). Recently, Wigmore and Lacey (Wigmore and Lacey, 1998) have shown that activation of group III mGluRs inhibits excitatory transmission onto midbrain dopamine neurons. Recent developments in selective pharmacological tools (Thomas et al., 2001;Flor et al., 2002;Marino et al., 2003b) and the availability of knockout mice make it possible to determine with a high degree of certainty which group III mGluR mediates a particular physiological effect. Based on the potential role of excitatory inputs to the SNc in determining the normal physiology of these cells, as well as the neurodegeneration associated with PD, we set out to pharmacologically characterize this group III mGluR-mediated modulation of transmission.

Materials and Methods

Compounds

(-) Bicuculline methobromide (bicuculline), (2S)-3-[[(1S)-1-(3,4-chlorophenyl)ethyl]amino-2-hydroxypropyl]phosphinic acid (CGP55845), (R,S)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), (S)-3,4-Dicarboxyphenylglycine ((S)-3,4-DCPG), N-Phenyl-7-(hydroxymino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) were obtained from Tocris (Ballwin, MO). L-(+)-2-amino–4-phosphonobutyric acid (L-AP4) was obtained from Alexis/Qbiogene Inc (Carlsbad, CA). All other materials were obtained from Sigma (St. Louis, MO).

Animals

All animals used in these studies were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Merck Research Laboratories Institutional Animal Care and Use Committee (IACUC) approved all studies described in this manuscript and experimental protocols were in accordance with all applicable guidelines regarding the care and use of animals. Animals were housed in an AALAC International approved facility with free access to food and water.

Slice Preparation

All electrophysiology experiments were performed on normal parasagittal slices from either 15 to 20-day-old Sprague Dawley rats (Taconic, Germantown, NY) , or 7 to 12 days old mice. The mGluR4 knockout mice (Gprc1d) (Pekhletski et al., 1996) were maintained in a colony at Emory University. Wild type 129X1/SvJ mice were obtained from Jackson Labs (Bar Harbor, ME). Animals were killed by decapitation and brains were rapidly removed and submerged in an ice-cold choline replacement solution (CRS)

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containing (in mM): choline chloride 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $MgSO_4$ 8, glucose 10, and $NaHCO_3$ 26, equilibrated with 95% O_2/5% CO_2. The brain was glued to the chuck of a vibrating blade microtome (Leica Microsystems, Nussloch GmbH) and 250 μm thick slices were obtained. Slices were immediately transferred to a holding chamber containing normal artificial cerebrospinal fluid (ACSF) (in mM): 124 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 2 $CaCl_2$, 20 glucose, and 26 $NaHCO_3$, equilibrated with 95% O_2/5% CO_2 that was maintained at room temperature. In all experiments 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid were included in the choline chloride buffer and in the holding chamber ACSF.

Electrophysiology

Whole-cell patch-clamp recordings were obtained as described previously (Valenti et al., 2003). During recording, slices were maintained fully submerged on the stage of a brain slice chamber at room temperature and perfused continuously with equilibrated ACSF (2-3 ml/min). Neurons were visualized using a differential interference contrast microscope and an infrared video system. Patch electrodes were pulled from borosilicate glass on a two-stage puller and had resistances in the range of 3-7 MΩ when filled with internal solution. In order to characterize the electrophysiological proprieties of the neurons, a potassium gluconate internal solution was used (in mM): potassium gluconate 125, NaCl 4, NaH_2PO_4 6, $CaCl_2$ 1, $MgSO_4$ 2, BAPTA-tetrapotassium salt 10, HEPES 10, Mg-ATP 2, Na_2-GTP 0.3. For all voltage clamp experiments, the internal solution consisted of (in mM): 140 cesium methane sulphonate, 16 HEPES, 10 NaCl, 2 EGTA, 2 MgATP, 0.2 mM Na_2-GTP.

All recordings were done using HEKA EPC9 patch clamp amplifiers (HEKA Elektronik, Lambrecht/Pfalz, Germany). Excitatory postsynaptic currents (EPSCs) were evoked by electrical stimulation, in the presence of blockers of $GABA_A$ (25 μM Bicuculline methobromide), and $GABA_B$ (100 nM CPG 55845) receptors. Bipolar tungsten stimulation electrodes were placed either locally in the SNc ~ 100 μm rostral to the recording site or in the STN. No difference was observed between these 2 groups and the data were combined. EPSCs were evoked from a holding potential of –70 mV by single pulses that ranged from 3-15 V, 200-400 μsec, delivered once every 30-60 seconds. Monosynaptic EPSCs for failure analysis were stimulated locally through a patch pipette filled with ACSF. These parameters were varied in order to optimize EPSC amplitude and stability.

The SNc was recognized as a cell-dense region dorsal to the SNr in slices corresponding to figures 82-85 of the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1998), and 108-117 of the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001).

All compounds were typically made in a 1000X stock and diluted into the ACSF immediately before use. L-AP4 and (S)-3,4-DCPG were made daily; all other compounds were aliquoted and stored at -20^{o} C. Compounds were applied to the bath using a three way stopcock. Agonists were always applied for 10 minutes in order to achieve a plateau concentration, while antagonists were applied 5 min prior to agonists and maintained in the bath during agonist application.

Statistics

Individual comparisons were made using a Student's, or paired Student's t-test as appropriate. In cases were multiple comparisons were evaluated, an ANOVA was employed followed by post hoc testing with Fisher's LSD test. All data are reported as mean ± standard error of the mean.

Results

Characterization of the Excitatory Transmission in DA neurons of the SNc.

To test the hypothesis that activation of group III mGluRs modulates transmission at the subthalamo-nigral synapse, we employed whole cell patch clamp recording from dopaminergic neurons in the rat SNc. Consistent with previous reports (Richards et al., 1997), we observed a homogeneous population of neurons with distinct electrophysiological properties including low frequency spike firing, large afterhyperpolarizations, and a prominent time dependent inward rectification produced by the injection of hyperpolarizing currents (Figure 1A).

Excitatory postsynaptic currents (EPSCs) were evoked as described in the methodology section. SNc dopaminergic neurons receive glutamatergic projections from the STN as well as a mix of glutamatergic and cholinergic inputs from the pedunculopontine nucleus (Lavoie and Parent, 1994;Charara et al., 1996). We attempted to favor the glutamatergic inputs from the STN by stimulating rostral to the recording site or within the STN. Single shock electrical stimulation elicited a fast inward current, showing a short and constant latency that attained peak amplitude within approximately 10 msec (Figure 1B). The glutamatergic nature of these EPSCs was confirmed by bath application of the AMPA/kainate-selective antagonist, CNQX (20 μM). CNQX produced a complete block of the synaptic response (predrug amplitude: -260.94 ± 56.37 pA, mean ± SEM; 20 μM CNQX amplitude: -19.98 ± 5.14 pA, mean ± SEM; n=5 $p<0.05$, paired t-test) (Fig 1B-D) suggesting that under these conditions these EPSCs are glutamatergic and predominantly due to activation of AMPA or kainate receptors.

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Activation of group III mGluRs Inhibits Excitatory Transmission in the SNc.

Wigmore and Lacey (Wigmore and Lacey, 1998) have previously shown that the activation of group III mGluRs caused inhibition of excitatory postsynaptic potentials (EPSP) in a dose-dependent manner. This depression of the EPSP was reversible and evident at a low concentration (0.3-30 μM), in a range that suggested activation of high affinity receptors. We employed voltage clamp methods to confirm that activation of group III mGluRs modulates excitatory transmission in midbrain dopamine neurons. Application of the highly selective group III mGluR agonist L-AP4 (Bushell et al., 1995) produced a significant inhibition of the evoked EPSCs (Figure 2A) that reversed as the compound washed out of the bath (Figure 2B). The response to L-AP4 was dose-dependent (Figure 2C) with L-AP4 producing a maximal effect of 61.85 ± 2.82 % inhibition at 3 μM (predrug amplitude: -204.2 ± 34.31 pA, mean ± SEM; 3 μM L-AP4 amplitude: -82.73 ± 19.35 pA, mean ± SEM; n= 7; $p<0.05$, paired t-test). L-AP4 exhibits potencies at recombinant rat group III mGluRs of 0.2-1 μM at mGluR4, 0.6-0.9 μM at mGluR6, 160-1300 μM at mGluR7, and 0.7-0.9 μM at mGluR8 (Schoepp et al., 1999). Since mGluR7 is only activated by high concentration of L-AP4 and mGluR6 is not expressed at high levels in the brain (Nakajima et al., 1993), this effect is consistent with an action at either mGluR4 or mGluR8.

L-AP4 Inhibits Transmission by a Presynaptic Mechanism

Immunocytochemical studies have not detected significant levels of mGluR4 or 8 in the SNc, however in situ hybridization studies have reported low to moderate levels of mGluR4 and 8 expression in both the SNc, and the STN (Testa et al., 1994;Messenger et

al., 2002). In many brain regions, the group III mGluRs are found presynaptically localized where they modulate the release of neurotransmitter. Based on this, we would hypothesize that either mGluR4 or mGluR8 trafficked to the presynaptic STN terminals may mediate the L-AP4-induced inhibition of excitatory transmission in the SNc. In order to test this hypothesis, we attempted to determine if L-AP4 reduces excitatory transmission in the SNc by a pre-or postsynaptic mechanism. We first attempted to study the effect of L-AP4 on paired-pulse facilitation. In these studies, we observed a trend towards an increase in paired-pulse ratio in 3 out of 4 cells; however, a high degree of variability in the baseline paired-pulse ratio prohibited an accurate quantitative analysis of these data. We next attempted an analysis of tetrodotoxin-resistant miniature EPSCs, however the extremely low frequency of miniature EPSCs in this region made these studies impossible. Therefore, we employed an analysis of the effects of L-AP4 on failure of monosynaptic EPSCs (Malinow and Tsien, 1990). As described in the methods section, focal electrical stimuli were employed to evoke single fiber monosynaptic EPSCs that had a measurable failure rate (Figure 3A, 3B) (event probability: 0.79 ± 0.02, n=4). In contrast to the evokes EPSCs described above which are produced by the synchronized release of transmitter from many synapses, these events are monosynaptic. Thus, alterations in failure rate reflect changes in presynaptic release probability while alterations in amplitude reflect changes in postsynaptic receptor function. Application of 3 μM L-AP4 produced a significant increase in failures (L-AP4 event probability: 0.13 ± 0.08; n=4; $p<0.001$, paired t-test) (Figure 3C). The failure rate increased in all cells studied however in one cell the failure rate increased to 100%. Application of 3μM L-AP4 still produced a significant increase in failure rate when this non-responding cell was

excluded from the analysis (predrug event probability: 0.80 ± 0.03, L-AP4 event probability: 0.18 ± 0.1; n=3; $p<0.05$, paired t-test). When the mean amplitude of events from these three cells was compared with the pre-drug amplitude, no significant effect was observed (Figure 3D) (predrug amplitude: -21.0 ± 3.1 pA, mean ± SEM; 3 μM L-AP4 amplitude: -14.8 ± 1.2 pA, mean ± SEM; n=3; $p>0.1$, paired t-test). This increase in failure rate without effect on mean amplitude is consistent with a presynaptic site of action (Malinow and Tsien, 1990) suggesting that L-AP4 decreases excitatory transmission through a presynaptic mechanism of action.

Pharmacology of the L-AP4 Mediated Inhibition of Excitatory Transmission in the Rat SNc

As discussed above, the different potency of L-AP4 at group III receptors suggests that the group III mGluR-mediated inhibition of excitatory transmission in the SNc is consistent with mGluR4 or mGluR8. Since mRNA for both mGluR4 and mGluR8 are expressed in the STN, the likely source of the glutamatergic afferents stimulated in these studies, it is possible that either receptor mediates this effect. We employed available pharmacological tools to attempt to distinguish between these two receptors. CPPG, a group III mGluR-preferring antagonist (Toms et al., 1996) was employed to confirm that this effect of L-AP4 is mediated by group III mGluRs. Pre-application of 100 μM CPPG inhibited the L-AP4-induced suppression of excitatory transmission in SNc neurons (predrug amplitude: –193.54 ± 29.3 pA, mean ± SEM; 100 μM CPPG amplitude: –175.6 ± 34.3 pA, mean ± SEM; n=5; $p>0.05$, paired t-test) (Figure 4C). In order to test for the involvement of mGluR8, we employed the mGluR8-selective agonist

(S)-3,4-DCPG. (S)-3,4-DCPG exhibits potencies at recombinant human group III mGluRs of 8.8 μM at mGluR4, 3.6 μM at mGluR6, >100 μM at mGluR7, and 31 nM at mGluR8 (Thomas et al., 2001). Application of 300 nM of (S)-3,4-DCPG, a concentration 10 fold higher than the EC_{50} of this compound at recombinant mGluR8 produced no effect on the excitatory transmission at SNc (predrug amplitude: –196 ± 14.7 pA, mean ± SEM; 300 nM (S)-3,4-DCPG amplitude: –184 ± 7.1 pA, mean ± SEM; n= 3; $p>0.1$, paired t-test) (Figure 4A-C). This result suggests that mGluR8 does not play a role in modulating transmission at the rat STN-SNc synapse, therefore we hypothesized that mGluR4 mediates the effect of L-AP4 in modulating excitatory transmission onto dopaminergic neurons in the SNc. In order to test this hypothesis, we employed the recently characterized highly selective allosteric potentiator of mGluR4, PHCCC (Flor et al., 2002;Marino et al., 2003b). PHCCC potentiates recombinant rat mGluR4 with a EC_{50} of 3.7 μM and exhibits antagonist actions at all other group III mGluRs (Marino et al., 2003b). Therefore PHCCC represents a selective and novel tool for studying the physiology of mGluR4. We employed PHCCC to confirm the hypothesis that mGluR4 modulates excitatory transmission in SNc. We choose a submaximal 300 nM dose of L-AP4 based on our dose-response study. Application of 300 nM L-AP4 produces a small, yet significant reduction in excitatory transmission (predrug amplitude: -164.4 ± 32.2 pA, mean ± SEM; 300 nM L-AP4 amplitude: -120.85 ± 24.57 pA, mean ± SEM; n= 9; $p< 0.05$, paired t-test). Application of the vehicle 1% DMSO (data not shown) or 30 μM PHCCC alone had no effect on the subthalamo-nigral transmission (Figure 5C). However, when co-applied with 300 nM L-AP4, 30 μM PHCCC produced a significant potentiation of the L-AP4-induced inhibition of the excitatory transmission (predrug

amplitude: -208.83 ± 68.36 pA, mean ± SEM; 300 nM L-AP4 + 30 μM PHCCC amplitude: -124.38 ± 47.71 pA, mean ± SEM; n= 6; p< 0.025, paired t- test) (Figure 5A-C). Taken together with the potency of L-AP4, and the lack of effect of (S)-3,4-DCPG, these data suggest that the L-AP4 modulation of synaptic transmission at the rat STN-SNc synapse is mediated by an activation of mGluR4.

Group III mGluR-Mediate Modulation of Transmission in mGluR4 knockout mice.

Our pharmacological studies suggest that mGluR4 modulates glutamatergic transmission in the SNc. In the attempt to provide further confirmation, we examined the group III mGluR-mediated modulation of excitatory transmission in wild type and mGluR4 knock out mice. We first characterized dopamine neurons of 129X1/SvJ wild type mice in current clamp mode (Figure 6A). Mouse neurons of the SNc exhibited electrophysiological properties consistent with dopaminergic neurons including low frequency spike firing, large afterhyperpolarizations, and a time dependent inward rectification produced by the injection of hyperpolarizing currents.

EPSCs were evoked by electrical stimulation identical to that used in the rat experiments (Figure 6B). Consistent with our findings in the rat, L-AP4 inhibited excitatory transmission in the SNc of slices prepared from 129X1/SvJ mice. L-AP4 produced a maximal inhibition of transmission at a concentrations of 1-10 μM (predrug amplitude: -190.8 ± 45.84 pA, mean ± SEM; 10 μM L-AP4 amplitude: -94.32 ± 25.46 pA, mean ± SEM; n= 5; p<0.05, paired t-test) (Figure 6B-D). The effect was dose-dependent, and reversible (Figure 6C-D).

Surprisingly, in mGluR4 knockout mice application of 10 μM L-AP4 produced a 62 ± 9% inhibition of the excitatory transmission (predrug amplitude: -130.48 ± 36.31 pA, mean ± SEM; 10 μM L-AP4 amplitude: -50.94 ± 28.25 pA, mean ± SEM; n=5; $p<0.05$ paired t-test) (Figure 7A, 7E), that reverse with the drug washed (Figure 7C). This suggests that another Group III mGluR modulates excitatory transmission in the mGluR4 knockout mouse. Based on the high potency of this effect, mGluR8 is the most likely receptor to mediate inhibition of the EPSCs. Consistent with this, application of 300 nM (S)-3,4-DCPG produced a significant inhibition of excitatory transmission in the SNc in slices from mGluR4 knockout mice (predrug amplitude: -140.5 ± 40.98 pA, mean ± SEM; 300 nM of (S)-3,4-DCPG amplitude: -78.25 ± 34.87 pA, mean ± SEM; n= 4; $p<0.05$ paired t-test) (Figure 7B, D, E).

Based on the strength of our pharmacological data in the rat, the finding that both L-AP4 and (S)-3,4-DCPG reduce excitatory transmission in the mGluR4 knockouts suggests several possible explanations. First, since we found it necessary to perform these studies in younger mice, the possibility exists that the observed difference is due to a developmental regulation of mGluR8. We therefore tested for the effect of (S)-3,4-DCPG in younger (p7-12) rats. These studies failed to find a significant effect of (S)-3,4-DCPG on EPSC amplitude (predrug amplitude: -77.2 ± 10.7 pA, mean ± SEM; 300 nM of (S)-3,4-DCPG amplitude: -60.6 ± 7.0 pA, mean ± SEM; n= 5; $p>0.05$ paired t-test) suggesting that the difference between rat and mouse is not due to a difference in developmental expression of mGluR8. It is also possible that a species difference exists between rats and mice such that mGluR8 alone, or both mGluR4 and mGluR8 modulate excitatory transmission in the mouse SNc. Finally, it is possible that the knockout of

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mGluR4 leads to a developmental compensatory upregulation of mGluR8 at this synapse. To distinguish between these hypotheses, we performed a set of pharmacological studies in wild type mice. We first employed the mGluR8 selective agonist (S)-3,4-DCPG and found that activation of mGluR8 decreases evoked EPSCs amplitude in the SNc. As shown in figure 7E, 300 nM (S)-3,4-DCPG produced a significant reduction in EPSC amplitude (predrug amplitude: - 170.12 $\pm$ 51.83 pA, mean $\pm$ SEM; 300 nM (S)-3,4-DCPG amplitude: - 130.58 $\pm$ 36.3 pA, mean $\pm$ SEM; n= 5; $p<0.05$, paired t-test). These results suggest that mGluR8 inhibits excitatory transmission in the SNc of control mice, indicating that a species difference exist between rat and mouse. However, the fact that activation of mGluR8 inhibits transmission at this synapse does not exclude the possibility that mGluR4 could also play a modulatory role in the mouse SNc. Therefore we employed the mGluR4 potentiator PHCCC in order to determine the role of mGluR4 in this effect.

As observed in slices from rats, application of 30 μM PHCCC alone had no significant effect on excitatory synaptic transmission in the SNc (predrug amplitude: -110.5 $\pm$ 45.6 pA, mean $\pm$ SEM; μM PHCCC amplitude: -118.2 $\pm$ 46.4 pA, mean $\pm$ SEM; n= 4; $p>0.05$, paired t-test). Co-application of 30 μM PHCCC and a submaximal dose of L-AP4 (300 nM) produced a dramatic potentiation of L-AP4-induced inhibition of EPSC amplitude (predrug amplitude: -199.67 $\pm$ 39.54 pA, mean $\pm$ SEM; 300 nM L-AP4 and 30 μM PHCCC amplitude: -115.87 $\pm$ 35.22 pA, mean $\pm$ SEM; n= 6; $p<0.05$, paired t-test) when compared with 300 nM L-AP4 alone (predrug amplitude: - 136.44 $\pm$ 15.02 pA, mean $\pm$ SEM; 300 nM L-AP4 amplitude: -102.94 $\pm$ 14.71 pA, mean $\pm$ SEM; n= 5; $p<0.05$, paired t-test) (Figure 8A-C), indicating that both mGluR8 and mGluR4 play a

role in modulating the excitatory transmission in the SNc of mice. Interestingly, the effect of (S)-3,4-DCPG observed in mGluR4 knockout mice was significantly greater than the effect observed in the wild type controls (ANOVA, Fisher's LSD $p<0.05$). While this finding must be interpreted with caution, it raises the interesting possibility that a functional compensation occurs in response to the loss of mGluR4 in the knockout mouse.

Discussion

In this study we employed whole cell patch clamp recording methods to investigate the pharmacology of group III mGluR modulation of excitatory transmission in the SNc. We found that in the rat, L-AP4-induced inhibition of transmission is mediated through a presynaptic mechanism by a receptor with a pharmacological profile consistent with mGluR4. Interestingly, when we attempted to confirm this finding using knockout mice, we discovered an apparent species difference. In the mouse both mGluR4 and mGluR8 appear to play a role in modulation of excitatory transmission in the SNc

Based on the high potency of L-AP4 in inhibiting excitatory transmission in the SNc, mGluR4 and mGluR8 are the most likely receptors to mediate the effects observed in these studies. The anatomical localization of mGluR4 mRNA in the rat (Testa et al., 1994;Messenger et al., 2002) and protein in both the rat and mouse (Bradley et al., 1999;Corti et al., 2002) has been previously described. Within the basal ganglia, mGluR4 immunoreactivity is observed at high levels in the globus pallidus, where it has been demonstrated to exist on presynaptic terminals that originate from the striatum(Bradley et al., 1999). In addition, there is a significant mGluR4-positive staining observed in the substantia nigra pars reticulata (Bradley et al., 1999;Corti, et al., 2002). To date there has not been an immunocytochemical study that has reported the distribution of mGluR8 within the basal ganglia. However, in situ hybridization studies have found significant expression of both mGluR4 and mGluR8 in neurons of the rat STN (Testa et al., 1994;Messenger et al., 2002).

Since the STN represents the most likely source of excitatory afferents stimulated in these studies, either mGluR4 or mGluR8 may be appropriately expressed and trafficked to presynaptic STN terminals to mediate this response. Our pharmacological studies in rat strongly support the hypothesis that mGluR4 mediates the L-AP4-induced inhibition of excitatory transmission in the SNc. The lack of immunoreactivity in the SNc may be due to low but functional levels of mGluR4 that escape immunocytochemical detection. Alternatively, it is possible that the synapses stimulated in these studies are predominately outside the SNc. Dopamine neurons are known to send dendrites deep within the SNr (Iribe et al., 1999) and STN afferents are known to associate with and terminate on dopaminergic dendrites within the SNr (Smith et al., 1990). Therefore, the pattern of mGluR4 immunoreactivity is consistent with modulation of the STN-SNc synapse.

In the mouse, immunocytochemical studies have revealed a distribution of mGluR4 that is identical to that observed in the rat (Bradley et al., 1999;Corti et al., 2002). As stated above, no immunocytochemical studies are available to determine the localization of mGluR8 protein in the rodent CNS. However, a comparison of [3H]L-AP4 autoradiography between wild-type and mGluR4 knockout mice revealed approximately 20% specific binding remaining in the SNr of mGluR4-deficient mice (Thomsen and Hampson, 1999). This high affinity specific binding is very likely due to the presence of mGluR8. Therefore, it is possible that both mGluR4 and mGluR8 protein is appropriately localized in the mouse to mediate the effects observed in the current studies. Future immunocytochemical studies will be necessary to confirm this hypothesis and to determine if mGluR8 is also present in the rat SNr.

While our present findings are consistent with what is know regarding the anatomical distribution of receptors in adult rats and mice, it is important to note that these studies have been performed in young animals. Recent work provides evidence for the developmental modulation of other mGluRs (Hubert and Smith, 2004). While it is not currently known, it remains possible that the group III mGluRs are developmentally regulated in the basal ganglia. Our finding that there is no difference in the effect of (S)-3,4-DCPG in rats between 7 and 20 days postnatal suggests that there is not a gross developmental switch between mGluR8 and mGluR4, however, further anatomical studies are needed to better characterize changes in the expression of these receptors during development.

We previously have suggested that activation of mGluR4 may provide a novel palliative intervention for the treatment of PD (Valenti, et al., 2003). From the stand point of neurodegeneration associated with PD, the finding that mGluR4 activation also depresses glutamatergic transmission in the SNc has interesting implication for this therapeutic strategy. The etiology of Parkinsonian neurodegeneration is complex and appears to involve a variety of factors including mitochondrial dysfunction and oxidative stress (Beal, 2000;Zhang et al., 2000). In addition, an indirect excitotoxic hypothesis has been proposed to explain in part the degeneration of dopamine neurons in PD (Albin and Greenamyre, 1992). According to this hypothesis, an inhibition of normal mitochondrial function leads to an energy deficit and subsequent inability of neurons to maintain a normal resting potential (Erecinska and Dagani, 1990). The resulting depolarization leads to a relief of the magnesium block of the NMDA receptors, and a state in which normal levels of glutamate become toxic (Novelli et al., 1988). According to the current

model of basal ganglia circuitry, the loss of striatal dopaminergic tone results in an excessive subthalamic glutamatergic drive that would be expected to exacerbate the neuronal loss associated with PD.

Consistent with this model, rodent studies have demonstrated that lesions of the STN decrease the 6-OHDA-induced degeneration of SNc neurons (Piallat et al., 1996). Furthermore, NMDA antagonists have been found to decrease the MPP+-induced degeneration of SNc neurons (Turski et al., 1991) as well as MPTP-induced degeneration of SNc neurons in primates (Zuddas et al., 1992). Interestingly, direct application of MK801 in the rat STN has been found to block the 6-OHDA-induced degeneration of SNc neurons (Blandini et al., 2001). Taken together, these studies suggest that a decrease in excitatory glutamatergic drive from the STN may be sufficient to provide some neuroprotective benefit in PD. This raises the exciting possibility that selective agonists acting at mGluR4 may provide both palliative benefit and a slowing of disease progression.

The apparent species difference observed in these experiments suggests that studies of dopamine receptor physiology and pathophysiology in different species must be interpreted with caution. For example, a brain penetrant mGluR4 agonist may provide both palliative and neuroprotective benefit in a rat model, but not in the more commonly used MPTP mouse model of neurodegeneration. Of course, the more import question of relevance to clinical PD must await a more detailed analysis of the distribution of these receptors in nonhuman primate and human brains.

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Figure Legends

Figure 1 - Characterization of EPSCs in dopaminergic neuron of the rat substantia nigra pars compacta. Figure shows an example of a current clamp recording from a dopamine neuron in the SNc (A). Dopamine neurons exhibit a characteristic time-dependent inward rectification, spike frequency adaptation, low spike frequency, broad spike width and a predominant afterhyperpolarization. Example traces (B) and representative time course (C) demonstrating electrically-evoked glutamatergic EPSCs recorded in SNc dopamine neurons. These inward currents exhibit a constant latency and are effectively blocked by application of the AMPA/kainate-selective antagonist CNQX (D) ($p<0.05$ paired t-test n=5).

Figure 2 - Activation of group III mGluRs modulates excitatory transmission in the rat substantia nigra pars compacta. Application of the highly selective group III agonist L-AP4 induced a dose dependent and reversible inhibition of excitatory transmission in the SNc. Representative traces (A) and time course (B) illustrating the rapid and reversible nature of this effect. C) Dose-response relationship of L-AP4 reveals a high potency effect that is maximal at 3 μM.

Figure 3 – The group III mGluR-mediated inhibition of transmission acts through a presynaptic mechanism. A) Representative average traces of all events (top) or failures (bottom) occurring in a 3 minute period before drug, and during the last 3 min of a 10 min application of 3 μM L-AP4. Note L-AP4 application produced little change in the monosynaptic EPSC waveform. B) Time course from the same experiment depicted in A, demonstrating an increase in the number of failures induced by L-AP4. Circled numbers represent regions averaged to produce traces shown in (A). Dotted line represents the cutoff that was used to determine if stimulus produced an event. This

cutoff represents a value of 2 standard deviations above the RMS noise and was used in choosing traces for part A, and in the probability and amplitude calculations in parts C and D. C) Summary data from 4 cells demonstrating a significant decrease in the probability of eliciting a response (i.e. increase in failure rate) produced by application of 3 μM L-AP4. * $p<0.001$ paired t-test. D) Summary data from the three cells that exhibited events in the presence of L-AP4 demonstrating a lack of significant effect on mean amplitude ($p>0.1$, paired t-test).

Figure 4 - Pharmacological studies of the effect of L-AP4 on excitatory transmission in the rat SNc. Representative traces (A) and time course (B) illustrating the lack of effect of a maximal concentration of the highly selective mGluR8 agonist (S)-3,4-DCPG 300 nM). C) Mean ± SEM data summarizing the CPPG-induced block of L-AP4 inhibition. The white bar represents mean ± SEM data from studies employing (S)-3,4-DCPG. The lack of effect suggests that mGluR8 does not play a major role in the L-AP4-induced inhibition of excitatory transmission in the SNc. (*$p<0.05$ paired t-test, n=5).

Figure 5 – PHCCC potentiates the L-AP4 – mediated inhibition of EPSCs in the Rat SNc. Example traces illustrating the effect of a submaximal dose of L-AP4 (A) and L-AP4 combined with PHCCC (B) on excitatory transmission in the SNc. C) Bar graph of mean ± SEM data demonstrating a significant potentiation of the effect of 300 nM L-AP4 by the selective mGluR4 potentiator PHCCC. (* significant difference compared to L-AP4 alone, $p<0.05$, paired t-test, n=6-9 cells/condition)

Figure 6 – Characterization of the group III mGluR - mediated excitatory transmission onto mouse midbrain DA neurons. A) Example of a current clamp characterization of an SNc dopamine neuron recorded in a brain slice from a 129X1/SvJ mouse. The physiology of these cells is consistent with the physiology of dopamine neurons recorded in the rat. B) Representative traces and (C) time course illustrating the effect of the group III mGluR agonist L-AP4 on excitatory transmission in the mouse SNc. Note the rapid and reversible inhibition produced by application of the agonist. D) dose-response relationship of the effect of L-AP4 on excitatory transmission in the SNc.

Consistent with the observation in slices from rats, activation of group III mGluRs produces an inhibition that reaches a maximum at low micromolar concentrations.

Figure 7 – Activation of mGluR8 plays a role in the group III mGluR-mediated modulation of excitatory transmission in the SNc of the mouse. Example traces (A-B) and time course (C-D) illustrating the effect of the non-selective group III mGluR agonist L-AP4 (A,C) and the selective mGluR8 agonist (S)-3,4-DCPG (B,D) on excitatory transmission in the wild type mouse SNc. Both compounds produce a rapid and reversible inhibition. E) Mean ± SEM data demonstrating that mGluR8 appears to play a functional role in modulating transmission in the SNc of both wild type and mGluR4 knockout mice. (*significant drug effect relative to pre-drug amplitude, $p<0.05$, paired t-test. †significant difference between 129X1/SvJ and mGluR4 knockout $p<0.05$, ANOVA, Fishers LSD).

Figure 8 - Activation of mGluR4 plays a role in the group III mGluR-mediated modulation of excitatory transmission in the SNc of the mouse. Example traces (A) and time course (B) illustrating the effect of a low concentration of L-AP4 (300 nM) combined with the selective mGluR4 potentiator PHCCC (30 μM). C) Mean ± SEM of data illustrating the significant potentiation of the effect of L-AP4 by PHCCC. (*indicates a significant drug effect relative to pre-drug amplitude, $p<0.05$ n=6-9 cells/condition paired t-test; † indicates a significant effect of PHCCC, $p<0.05$ n=6-9 cells/condition ANOVA, Fishers LSD).

Fig 1

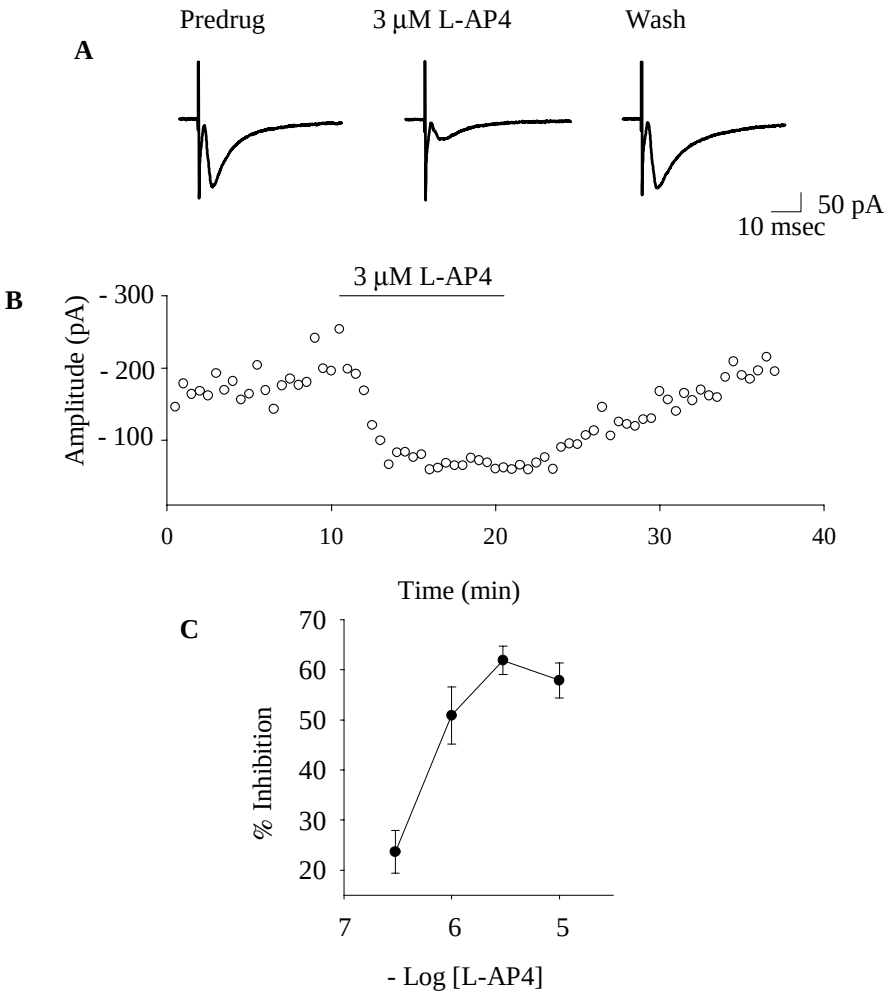

Fig 2

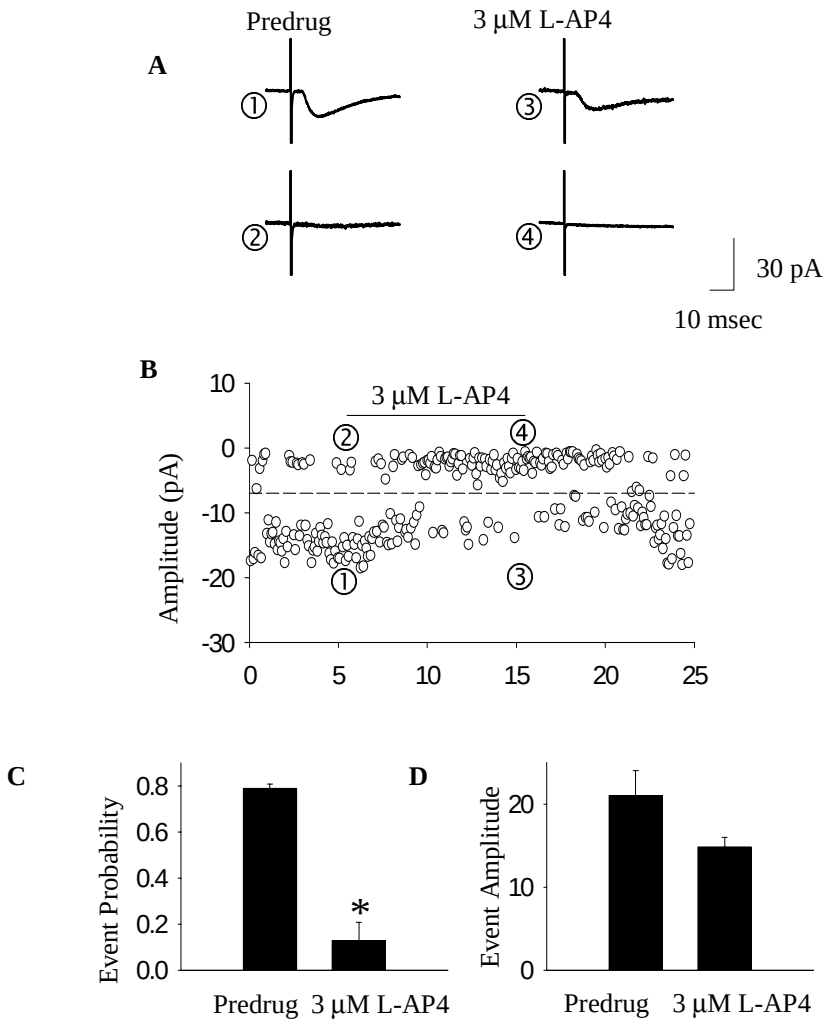

Fig 3

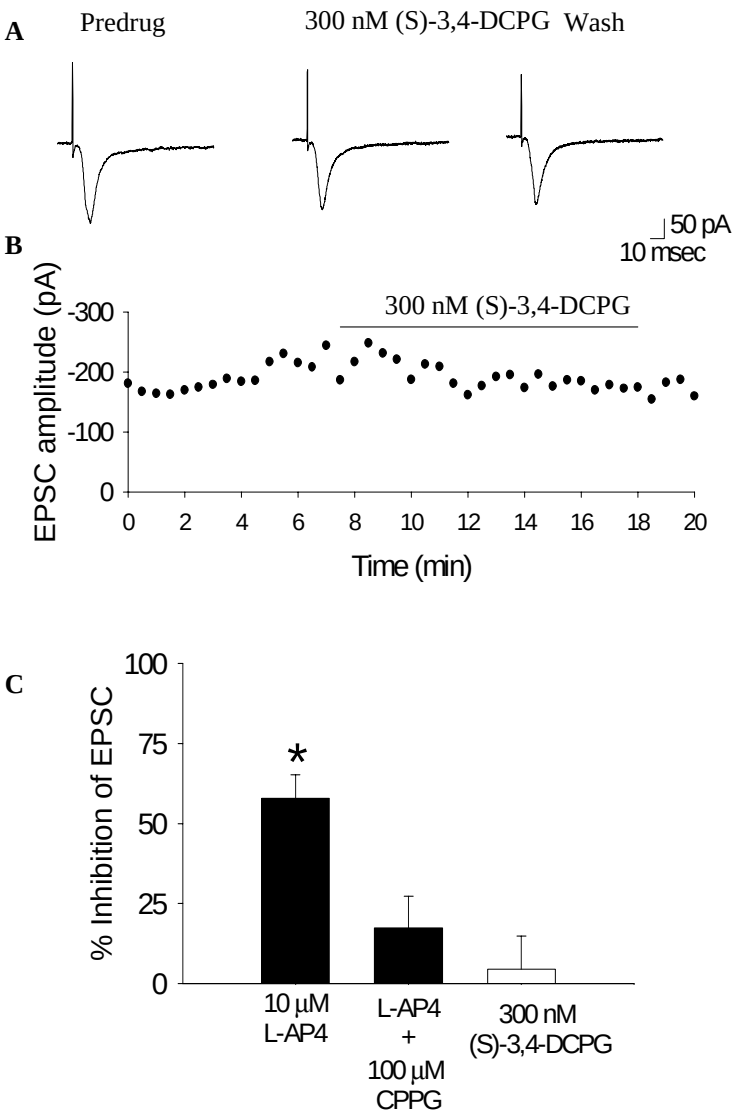

Fig 4

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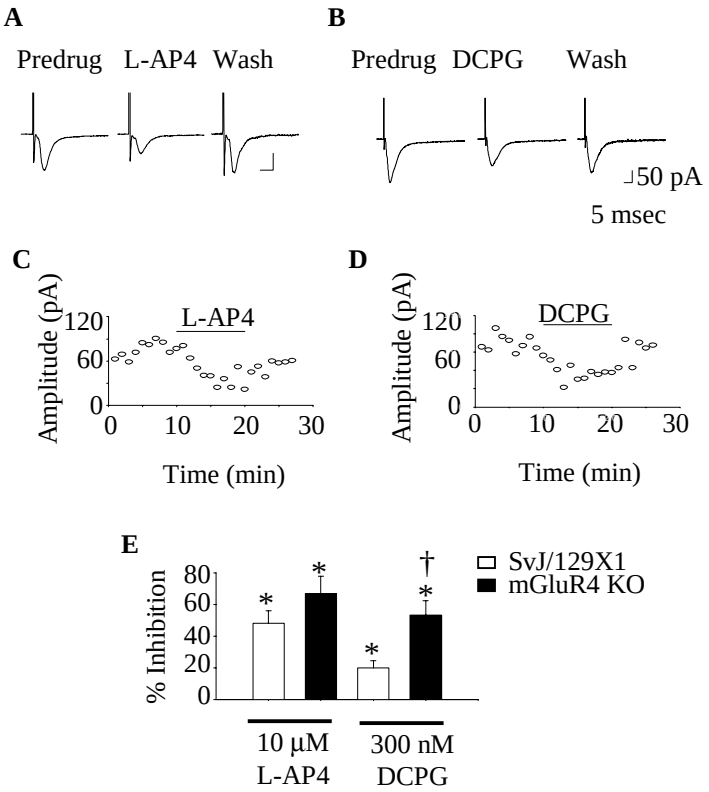

Fig 7

Fig 8