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In Parkinson's disea	ase (PD) nigrostriatal do	paminergic neurons	selective	ely die.		
Here we describe our	contributions toward un	derstanding basal	ganglia (E	3G)		
function and the sui	itability of metabotropic	glutamate recepto:	rs (mGluRs	;) as		
anatomical localizat	j PD, we employed immund tion of mGluBs 4, 2/3, 1a	and 5 in BG nucle	escribe tr			
electrophysiology to	o describe how mGluRs med	liate the effects of	f glutamat	e in rat		
brain slices. We tested the efficacy of mGluR drugs in relieving motor symptoms						
in hemi-parkinsonian monkeys, a non-human primate model of PD. We found that						
group III mGluRs are presynaptic on striatal-pallidal terminals and they reduce						
SNr synapse Groups I and II mGluBs also regulate BG function. Group II mGluBs						
mediate a presynaptic reduction of EPSCs in the SNr and group II agonist LY354740						
reverses catalepsy in (a rodent) model of PD. Post-synaptic group I mGluRs						
regulate BG output nuclei by both excitation and disinhibition, and are enriched						
in the globus pallic	lus. Furthermore, the lim	ited availability (of orally	available		
mGluR drugs confound	s our ability to ascerta	in wheter mGluRs w:	ili remain	a viable		
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INTRODUCTON

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. In PD there is a significant loss of nigrostriatal dopamine neurons that results in a series of neurophysiological changes that lead to a pathological excitation of the subthalamic nucleus (STN). The increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neurons in the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). This glutamate-mediated over excitation of the BG output nuclei ultimately leads to a "shutdown" of thalamocortical projections and produces the motor impairments characteristic of PD

Wichmann, 1997 #3].Unfortunately, as the disease progresses, the efficacy of traditional dopamine replacement therapy becomes severely diminished, and severe motor and psychiatric side effects can occur [Poewe, 1986 #14]. Because of this, a great deal of effort has been focused on developing new approaches for the treatment of PD. In these studies we are pursuing a novel therapeutic approach by targeting drugs acting at metabotropic glutamate receptors (mGluRs). Eight mGluR subtypes have been cloned (designated mGluR1-mGluR8) from mammalian brain. Each subtype is classified in one of three classes (I-III). Although initially we intended to study the Group III mGluRs, and mGluR4 in particular, we later discovered that the groups I and II mGluRs also play a crucial role in regulating BG function. Consequently, we have expanded our mGluR studies to include receptors from the other two groups. We have developed and characterized antibodies to mGluR 4a. We describe the anatomical distribution of mGluR4a and group I mGluRs. We describe the effect of selective mGluR agonists and antagonists on synaptic transmission in the SNr, STN, and GP all major basal ganglia loci. Finally, we evaluate the therapeutic potential of groups I, II, and III mGluR agonists and antagonists in hemi-parkinosnian monkeys. Since mGluRs play an important role in the modulation of BG function, the results of this study will provide a valuable contribution to our understanding of basal ganglia function and dysfunction.

BODY

Specific Aim I.

To localize mGluR4a and 4b receptors in rat and monkey basal ganglia by immunohistochemical techniques using subtype- and isoform- specific antibodies.

This aim is focused on the development and characterization of antibodies against mGluR4. The antibodies are used as a tool to reveal the anatomical distribution of these receptors in the basal ganglia of rat and rhesus monkey. We successfully developed and characterized a polyclonal antibody to the carboxyl-terminal portion of mGluR4a. An immunoblot (Figure 1) containing membrane protein from cells over-expressing mGluR2, mGluR5, mGluR7a, GluR4a, mGluR4b, and non-transfected Sf9 cells were probed with the affinity-purified mGluR4a antibody. Chemiluminescence 120kDa—



revealed a dark band at the predicted molecular weight for mGluR4a (~100 KD). Furthermore, it shows the exclusive recognition of membrane protein from cells expressing mGluR4a. Imunocytochemistry (ICC) was then used to determine the distribution and localization of mGluR4a in the rat

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brain. With 3,3'-diaminobenzidine (DAB) as the chromagen, light microscopy was used to show a distinct pattern of mGluR4a distribution in the basal ganglia (Figure 2). Very low levels of mGluR4a were detected in the striatum (STR). Virtually no staining was detected in the substantia nigra pars compacta (SNc). The substantia nigra pars reticulata (SNr), entopeduncular nucleus (EPN), and globus pallidus (GP), however exhibited densely labeled neuronal fibers. Staining was absent when primary antibody was omitted, and when

the antibody was pre-adsorbed to mGluR4a synthetic peptide prior to the ICC incubation. Furthermore, we used the technique of confocal microscopy (Figure 3) to demonstrate double labeling with mGluR4a antibodies and the presynaptic marker SV2. mGluR4a and SV2 immunoreactivity are clearly colocalized (arrows) along the outside of dendrites in the GP, suggesting a presynaptic localization of mGluR4a in the GP. Electron microscopy (Figure 4) was used to confirm the localization of this receptor to presynaptic terminals.



Figure 1

Figure 4. A-E: Electron micrographs demonstrating presynaptic mGluR4a immunoreactivity in the GP. Examples of mGluR4a axon terminals (asterisks) synapsing with dendrites (d) of cells in the GP. Scale bar = 400 nm in A-D; 350 nm in E.



Furthermore, quinolinate lesioning of the projecting neurons from the striatum to the GP induced a marked decrease in the ipsilateral but not contralateral (control) GP (Figure 5; red represents mGluR4a immunoreactivity). This suggests that such presynaptic localization is on striatopallidal terminals (Figure 5)[Bradley, 1999 #2]. Antibodies against mGluR4b, the other isoform were generated. Antibody characterization experiments

demonstrated that this immunoreactivity was not exclusively specific for mGluR4b. It recognized proteins in mGluR4 knock out brain tissue by both Western



immunocytochemistry. Our inability to demonstrate

specificity of the mGluR4b antibody precluded its use in further localization studies.

Immunocytochemical staining for group II mGluRs was performed in the basal ganglia of rhesus monkey. Electron microscopy was used to analyze the distribution of group II mGluRs in basal ganglia structures such as the STR (caudate nucleus, putamen, and accumbens core and shell). The majority of staining was seen in small unmyelinated axons, preterminal axonal segments of glutamatergic boutons and glial cell processes. A small population of dendrites of various sizes and spines also showed immunoreactivity, but labeled axon terminals were scarce. Pre-embedding immunogold revealed that only about 30% of the staining was membrane bound and of that, only about 5% was observed at synapses. From this we conclude that group II mGluRs are anatomically positioned to subserve pre- and post- synaptic functions in the monkey striatum under conditions that induce extrasynaptic spill over of glutamate. Specific Aim II.

Determine the effect of selective group III mGluR agonists and antagonists on synaptic transmission at the major excitatory synapses in the output nuclei of the basal ganglia.

Whole cell patch clamp techniques were used to record the effects of mGluR agonists and antagonists on synaptic transmission in the BG. Here we provide a thorough characterization of the roles that mGluRs play at the major synapses of the BG. In particular the roles of mGluRs in the SNr, STN, and the GP were studied.

The SNr is a primary output nucleus of the basal ganglia. A fine balance between excitatory and inhibitory inputs controls SNr function. The major excitatory input to GABAergic neurons in the SNr arises from glutamatergic neurons in the STN. In the SNr we showed (Figure 7) that group III mGluRs mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse [Bradley, 1999 #4]. In addition, group III mGluRs mediate a reduction in IPSC amplitude in the SNr [Wittmann, 1999 #3]. We have also shown that the group III mGluRs (mGluR4 and mGluR7) are presynaptically localized on striatal terminals in the GP, where they could reduce GABA realease (Wittmann 1999), and that activation of the group III mGluRs inhibits both GABAergic and glutamatergic transmission in the SNr (Marino 1999; Bradley, Marino et al. 2000). To fully understand the role of mGluR4, however, we carefully assessed the roles of other mGluR subtypes since glutamate will activate all receptors present in basal ganglia structures. Routine controls for the effect of other mGluRs has produced some interesting findings.

Group I and group II mGluRs play powerful roles in regulation of basal ganglia output. Group II mGluRs were found to mediate a presynaptic reduction of EPSCs in the SNr. Consistent with this finding is the observation that systemic administration of the highly selective group II mGluR agonist LY354740 reverses catalepsy in a rodent model of PD [Bradley, 1999 #9; Bradley, 1999 #4; Marino, 1999 #7; Bradley, 1999 #24]. Activation of group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons [Wittmann, 1999 #3; Marino, 1999 #1; Marino, 1999 #23]. These findings suggest that activation of group I mGluRs excite GABAergic projection neurons both by direct stimulation and by disinhibition. Immunocytochemical analysis at the light and electron microscopic levels reveal that both mGluRs 1 and 5 are localized post-synaptically at putative glutamatergic synapses in the SNr. mGluR1 appears to be the sole mediator of the group I mediated depolarization of inhibitory GABAergic neurons in the SNr. mGluR1 also mediates a decrease in synaptic transmission at excitatory synapses. This decrease in excitatory transmission

occurs by a presynaptic mechanism, as confirmed by the immunocytochemical data (Marino, Wittmann et al. 2001; Wittmann 2001).

Light presynaptic staining for mGluR1 was also observed at asymmetric synapses in the SNr. This is in agreement with the finding that activation of presynaptic group I mGluRs decreases inhibitory transmission in the SNr. The combination of excitation and disinhibition induced by group I mGluR activation could lead to a large excitation of the SNr projection neurons. This is likely to play an important role in the powerful excitatory control that the STN exerts on the BG output neurons of the SNr. [Marino, 2000 #18]

Figure 6.



EPSCs Stimulation of the STN evokes a glutamatergic EPSC in SNr neurons, which is modulated by activation of mGluRs. (A) Bath application of the group II selective agonist LY354740 (100 nM), or the group III selective agonist L-AP4 (C), inhibits evoked EPSCs. The group I selective agonist DHPG (500 μ M) had no effect. The non-NMDA glutamate receptor antagonist CNQX (20 μ M) completely blocked these currents indicating that they are glutamatergic EPSCs (data not shown) (B) Mean data demonstrating both group II and group III selective agonists inhibit synaptic transmission at the STN-SNr synapse. Data from representative neurons (A), or the mean + s.e.m. of data from 5 cells (B).



IPSPs Stimulation of inhibitory inputs evokes a GABAergic IPSC in SNr neurons, which is modulated by activation of mGluRs. (C) Bath application of the group I selective agonist DHPG (100 μ M), or the group III selective agonist L-AP4 (500 μ M) inhibits evoked IPSCs (E). The group II selective agonist inhibits synaptic transmission at GABAergic synapses on SNr neurons (D). Data from representative neurons (C), (D), and (E) or the mean + s.e.m. of data from 5 cells(F).

In addition to the direct excitatory actions of the SNr, mGluRs might also alter the activity of the SNr by modulating its primary source of glutamatergic excitation, the STN. The STN plays an important role in normal motor function and in PD by providing the major glutamatergic excitatory input to the basal ganglia output nuclei. We examined the direct effects of group I agonists on STN neurons and found that group I mGluRs, mGluR5, in particular mediates the depolarization of STN neurons [Awad, 1999 #6]. Stimulating electrodes were placed in the internal capsule (IC) for stimulating descending afferents and in the cerebral peduncle for stimulating ascending afferents. EPSCs were elicited in the STN in the presence of 10µM Bicuculline, and IPSCs were elicited in the presence of 20µM CNQX and 20µM L-AP4. Under IC stimulation, the group I selective mGluR agonist DHPG (100µM) caused a 34.3±3.3% reduction of EPSCs, the group II agonist LY354740 (100nM) caused a 43.5±6.8% reduction in EPSCs, and the group III agonist L-AP4 (1mM) caused a 80.9±6.7% reduction in EPSCs (Awad 2000).

Unlike the groups I or III mGluRs, group II mGluRs has no effect on excitatory transmission in the STN. Paired pulse studies suggest that mGluR1 and the group III mGluR-mediated effects are due to a presynaptic mechanism. If these receptors are involved in endogenous synaptic transmission in the STN, the data further supports the possibility that selective drugs targeting mGluRs may provide an alternative approach to the treatment of PD (Awad-Granko 2001).

Finally, the electrophysiological studies were extended to the GP. Previously we reported preliminary findings on the functional roles of group I mGluRs in the two most predominant cell types found in the GP. Group II and group III mGluR agonists were shown to have no effect on membrane potential in type II GP neurons. DHPG, a group I mGluR5 antagonist caused an increase in DHPG-mediated depolarization, and induced oscillations in membrane potential in type II GP neurons. MPEP, a mGluR5 antagonist caused an increase in DHPG-mediated depolarization, and induced oscillations in membrane potential in type II GP neurons. The mGluR1 antagonist, LY367385, suppressed DHPG-mediated depolarization in type II GP neurons (Maltseva 2000). Since the group I mGluRs 1 and 5 were found to frequently co-localize in the same neurons throughout the GP, and to produce the most interesting electrophysiological findings, we focused our most current studies on them. Both receptors can couple to the same effector systems, the purpose of their cellular co-expression remains unclear. We found that mGluRs 1 and 5 have distinct functional roles in type II neurons of the rat GP. Type II GP neurons form a large population of GABAergic projection neurons that are characterized by the presence of inwardly rectifying

current I_h, low threshold voltage-activated calcium current I_p and activity at rest. Although immunocytochemical analysis reveals a high degree of neuronal co-localization of the two group I mGluRs in the GP, activation of mGluR1 only directly depolarizes type II GP neurons. Interestingly, blockade of mGluR5 by a highly selective antagonist, MPEP, leads to the potentiation of the mGluR1-mediated depolarization in this neuronal sub-population. mGluR1 desensitizes upon repeated activation with the agonist in type II GP neurons and blocking mGluR5 prevents the desensitization of the mGluR1-mediated depolarization. Elimination of the activity of protein kinase C (PKC) by an application of 1 μ M bisendolylmaleimide or 1 μ M chelerythrine, both protein kinase C inhibitors, potentiates the mGluR1-mediated response and prevents the desensitization of mGluR1 in type II GP neurons, suggesting that the effect of mGluR5 on mGluR1 signaling may involve PKC. Together, these data illustrate a novel mechanism by which mGluR1 and mGluR5, members of the same family of G-protein coupled receptors, can interact to modulate neuronal activity in the rat GP (Poisik 2002; Poisik 2002).

Specific Aim III. To evaluate the therapeutic potential of group III agonists in hemi-parkinsonian monkeys.

This aim directly measures the efficacy of mGluR agonists on hemi-parkinsonian monkeys. Two rhesus monkeys were behaviorally conditioned to tolerate transfers from their home cages into the primate chair. We then carried out base line observations using an automated activity monitoring cage with eight sets of infrared beams. Activity is being measured by counting infrared (IR) beam crossings over a 20-minute period. The pattern of IR crossings is also stored on computer disk, and can be used to later analyze behavioral patterns such as rotational behaviors, the amount of time spent in the upright posture, etc. In addition, we used a computer-assisted behavioral observation method, by which an observer scores movements of individual limbs of the animal by pressing keys on a computer keyboard. A computer stores the timing and length of key presses. More than one key can be scored at any given time. This allows for the calculation of the ratio between the left and right arm movements and allows for the normalization of the data with respect to the left (Parkinsonian) side. An increase in the L/R ratio indicates improvements in parkinsonism. For assessment of more distal motor control, a Kluever-board technique was used for both upper extremities separately. For this, the monkey was placed in front of a plexi -glass board with 16 wells, into which raisins were placed. The time needed to empty all wells (or the number of wells emptied in 20 seconds) was scored. Finally, a behavioral rating scale

was completed on each experimental day, scoring the presence or absence of parkinsonian motor signs, and dyskinetic movements/stereotypes. After the initial behavioral observations, the monkeys were each treated with a single injection of MPTP (0.4 mg/kg) into their right internal carotid artery, following published protocols [Bankiewicz, 1986 #11].

In order to achieve a hemiparkinsonian state, both animals had to receive multiple MPTP injections followed by a fairly lengthy observation period to document the presence of parkinsonian signs and possible recovery. This cycle had to be repeated several times for each animal before a stable nigrostriatal leision was achieved. After a stable parkinsonian state was documented with the above-mentioned behavioral observation methods, the animals were surgically fitted with steel recording chambers directed at the GPi and SNr/STN to carry out intracerebral injections. In one animal the SNr/STN chamber was positioned to also give access to the ventricular system for i.c.v. injections.

While difficulties are unavoidable in this type of experiment, we made some progress in testing for therapeutic efficacy of group II agonists. The group II mGluR agonist LY354740 (100 μ M) injected in GPi resulted in a slight increase in the L/R ratio in one hemi-parkinsonian monkey. The same agonist produced no change in the overall activity when injected into the STN, and in the cerebral ventricular system (i.c.v.). Both subcutaneous and intramuscular injection of this drug induced vomiting, but no reasonable anti-parkinsonian effects. Repeated injections of the group I antagonist MPEP (2mmol/L) and the group II mGluR antagonist LY341495 (1 mmol/L) had no effect when injected into the STN. The initial i.c.v. administration of group III agonist R,S PPG (1 μ M) resulted in substantial antiparkinsonian effects, with an almost two-fold increase of the arm L/R ratio baseline (Fig. 7). This effect did not repeat and appears to have been an isolated incident.



Figure 7. Comparison of the antiparkinsonian effects of i.e.v. injections of mGluR active compounds in a hemiparkinsonian rhesus monkey, as expressed by the arm L/R ratio. LY379268 is a group II mGluR agonist R,S PPG is a group III mGluR agonist.

Primate studies are inherently slow, and these experiments have been technically challenging despite our expertise and concerted efforts. Multiple factors have confounded this important specific aim. A general problem in all of our monkey studies is the current scarcity of suitable Rhesus monkeys. We are currently experiencing wait times of between 2 and 6 months for shipments of animals. The primary reasons why many animals are excluded from this research are concerns about their health; the recent death of a worker at the Yerkes Regional Primate Center here at Emory has particularly emphasized the potential dangers of the simian Herpes-B agent. This and other potential zoonotic threats are being evaluated during a three-month quarantine period, which each animal has to undergo here at Emory. We feel that only those animals that can be assumed with a reasonable degree of certainty to not carry these simian viral diseases should be used in the experiments, because close contacts between the monkeys and their care-takers cannot be avoided and should be kept as safe as possible. Additional delays arise as consequence of the experimental paradigm itself. The recovery may be

obvious in as short a period as two weeks after application of the toxin, but in other cases, may take several months to occur. Consequently, the animals have to be observed for extended periods of time (at least three months, in our experience) before recovery can be excluded. The animals treated under this specific aim each had to undergo multiple MPTP injections (four and seven injections, respectively) before a stable hemi-parkinsonian state was accomplished. This has resulted in significant, but unavoidable delays.

In the previous and current year, we administered several different mGluR agonists and antagonists at different doses. Each experiment involves injections and extensive behavioral analysis as was initially described in the proposal. In the previous year we performed 6 experiments testing the effects of different doses of R,S- PPG in 2 monkeys. We injected R,S- PPG, the group III agonist in the ventricular system at multiple doses. 1 μ M, 100 μ M, 1mM, and 10mM produced zero improvement in parkinsonian signs. LY379268, the group II agonist was given i.c.v. at 5 μ M, 3 μ M, and 1 μ M i.c.v. with no measureable effect. More recently we have tested LY379268 s.c. at 1 and 3 mg/kg in two different monkeys seen either no effect or negative side effects, including reproducible vomiting within minutes after administering the drug.

In aim 2 we discovered that mGluR 1 inhibited the excitatory transmission in the output nuclei in rats. Hence, MPEP or saline was administered to 1 monkey. MPEP is a very good group I mGluR antagonist that shows subtype-specificity for mGluR5. MPEP was given i.m. at doses of 0.1 mg/kg and 0.5 mg/kg. A vehicle injection of physiological saline showed no effect. The lowest dose of MPEP (0.1 mg/kg) showed no improvement of parkinsonism. The higher dose (0.5 mg/kg) produced disturbing side effects. These side effects included sleepiness, laying down, and hunching over, with zero improvement in parkinsonism. These findings are unexpected and somewhat disappointing given all of the promising electrophysiological and anatomical data that we have reported. We followed up the previous MPEP experiments, this time in the STN at a dose of 2 mmol/L, which also produced no change in overall motor activity. Additionally, we tested LY354740, a group II agonist at doses of 10 μ M,100 μ M and 1 mM in the SNr, the STN, and the GP. Neither dose produced an effect. A new group II agonist LY314593 was administered in the SNr at doses as high as 10 and 100 mM, with still no observable effects.

Finally we developed and validated a new technique for the on-line detection of glutamate in microdialysis samples in awake animals. This method is based on an enzymatic assay using fluorescence detection of glutamate. We demonstrate that this method is highly specific and sensitive for glutamate

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(detecting approximately 0.5μ M/L glutamate). This technique is useful because it can be used to detect changes in glutamate levels evoked by physiologic or pharmacologic manipulations in the primate brain (Galvan and Wichmann 2002).

KEY RESEARCH ACCOMPLISHMENTS

Anatomy:

- Antibodies to mGluR4a were developed, characterized, and used to describe the anatomical distribution and localization of mGluR4a in rat brain.
- Antibodies to group I mGluRs were thoroughly characterized and used to demonstrate that mGluR 1 and 5 are postsynaptic at putative glutamatergic synapses in the SNr, and that mGluR1 is presynaptic at asymmetric synapses in the SNr.
- Group II mGluRs are anatomically positioned to subserve pre- and post- synaptic functions in the monkey striatum under conditions that induce extrasynaptic spill over of glutamate.

Electrophysiology:

- Group III mGluRs mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse.
- Group III mGluRs mediate a reduction in IPSC amplitude in the SNr.
- Group II mGluRs mediate a presynaptic reduction of EPSCs in the SNr.
- Group II agonist LY354740 reverses catalepsy in an animal model of PD.
- Activation of group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons.
- Group I mGluRs, particularly mGluR5, mediate the depolarization of STN both by excitation and by disinhibition of output projection neurons.
- mGluR1a alone mediates the group I depolarization of inhibitory GABAergic SNr neurons.
- We demonstrated that activation of mGluR 1 inhibits glutamatergic transmission in the SNr.
- Activation of presynaptic group I mGluRs reduces inhibitory transmission in the SNr.
- Activation of groups I or III mGluRs inhibits excitatory transmission in the rat subthalamic nucleus.
- Group I mGluRs, especially mGluR 5 mediates STN neuron depolarization.

- Group I selective agonist DHPG reduces EPSCs in the STN.
- Group III agonist L-AP4 reduces EPSCs in the STN.
- Blockade of mGluR5 eliminates desensitization of mGluR1 in type II GP neurons.
- PKC modulates the DHPG-induced activation of mGluR1 in type II GP neurons.
- Blockade of PKC prevents desensitization of mGluR1 in type II GP neurons.

Behavior:

Two rhesus monkeys have achieved a stable hemi-parkinsonian state. They were behaviorally trained to tolerate transfers from their home cage into a primate chair. They were given various intracerebral and intraventricular, and intramuscular injections of mGluR agonists and antagonists to measure the efficacy of these drugs on the symptoms of parkinsonism.

- Group II mGluR agonist LY354740 (100 μM) injected in GPi resulted in a slight increase in the L/R ratio in one hemi-parkinsonian monkey. The same agonist produced no change in the overall activity when injected into the STN, and in the cerebral ventricular system (i.c.v.). Both subcutaneous and intramuscular injection of this drug induced vomiting, but no reasonable antiparkinsonian effects.
- Group II mGluR antagonist LY341495 (1 mmol/L) had no effect when injected into the STN.
- LY341495 produces no effect in the SNr at 10 μM,100 μM, 10 mM and 100 mM.
- Group I antagonist MPEP (2mmol/L) had no effect in the STN.
- MPEP was also given i.m. at doses of 0.1 mg/kg and 0.5 mg/kg. 0.5 mg/kg produced adverse side effects, including sleepiness, laying down, and hunching over, with zero improvement in parkinsonism.
- MPEP in the STN at a dose of 2 mmol/L produced no effect.
- Group III agonist R,S PPG (1 µM), i.c.v. resulted in substantial antiparkinsonian effects, with an almost two-fold increase of the arm L/R ratio baseline (Fig. 1). In subsequent experiments this effect did not repeat and appears to have been an isolated incident.
- R,S- PPG, the group III agonist in the ventricular system at multiple doses. 1 µM, 100µM, 1mM, and 10mM produced zero improvement in parkinsonian signs.
- LY379268, the group II agonist was given at 5 μM, 3 μM, and 1 μM i.c.v. with no effect.

- LY379268, s.c. at 1 and 3 mg/kg produce either no effect or negative side effects, including vomiting.LY354740, a group II agonist at doses of 100 µM and 1 mM in the SNr.
- LY354740 in the SNr, GPi, and the STN at 1 mM, 10 μM, and 100 μM produces no effect.
- A new enzymatic assay designed to detect short term changes in glutamate levels evoked by physiologic or pharmacologic manipulations in the primate brain was developed and validated.

REPORTABLE OUTCOMES (The following list includes all publications and meeting abstracts that were generated from the research effort.)

Book Chapters

- Marino, Michael J., *et al.* Localization and Physiological Roles of Metabotropic Glutamate Receptors in the Indirect Pathway. In Movement Disorders, M. DeLong, A.M. Graybiel, S.T. Kitai, eds. (2000).
- Poisik, Olga, et al. Metabotropic Glutamate Receptors in the globus pallidus. In IBAGS VII, L.F.B. Nicholson and R.L.M. Faull (eds) Kluwer Academic/Plenum Press: New York, pp. 233-242.

Papers and Manuscripts

- Bradley, S.R., *et al.*, Activation of Group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia nigra pars reticulata. *Journal of Neuroscience*, 2000. 20(9): p.3085-3094.
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CONCLUSIONS

Our studies addressed three areas of basal ganglia function with therapeutic implications for Parkinson's disease: anatomy, physiology, and pharmacology. First, we have successfully carried out a series of high resolution anatomical studies to show that 1) the group III receptor, mGluR4a is found in the STR, SNr, and EPN, key basal ganglia structures involved in the manifestation of Parkinsonian symptoms; 2) the GP contains a high concentration of pre-synaptic mGluR4a neuronal fibers that project from the striatum. This is significant because it provides physical evidence to support the hypothesis mGluR4a can serve as a presynaptic heteroreceptor involved in regulating GABA release(Desai 1991; Calabresi 1992; Stefani 1994); 3) mGluR2/3, a group II mGluR is localized extrasynaptically in monkey striatum. Such localization subserves pre- and postsynaptic functions under physiological conditions that induce extrasynaptic spillover of glutamate (Iskhakova 2002). In general the group II mGluRs are presynaptic in rat STN. mGluRs 1 & 5 are postsynaptic in the SNr, and are localized to some axon terminals forming symmetric synapses in small unmyelinated axons (Marino, Wittmann et al. 2001). However, one limitation of the studies is that the localization of staining depends on the specificity of the antibodies, and it is impossible to rule out some non-specific reactions in tissue sections. Although western blot analysis revealed that the mGluRs 4a, 2/3, 1a, and 5 antibodies selectively and specifically recognize mGluR4a, this remains an important caveat.

To extend the anatomy, we also performed the physiological studies which overall showed: 1) activation of Group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons; 2) Group II mGluRs mediate a presynaptic reduction of EPSCs in the SNr; 3) Group III mGluR agonists mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse and a reduction in IPSC amplitude in the SNr; 4) that activation of groups I or III mGluRs reduces the excitatory glutamatergic transmission in the STN, and that this reduction is mediated by a presynaptic mechanism; 5) that activation of group I mGluRs reduces glutamatergic synaptic transmission in GABAergic SNr neurons and that this effect is also mediated by a presynaptic mechanism. We believe each of these findings have important potential therapeutic implications for developing novel treatments for PD. The ability of group II and group III mGluRs to modulate the excitatory transmission of glutamate at the STN-SNr synapse, combined with the data obtained on the ability of group I mGluRs to excite BG output neurons and the anatomical findings all provide compelling evidence that the development of novel therapeutic agents that target the specific receptors at these synapses could provide relief from the symptoms of PD. Remarkably, we showed that the Group II agonist LY354740 reverses catalepsy in a rodent animal model of PD.

Finally, our studies attempted to extend the analysis to a direct therapeutic study in nonhuman primates model of PD. These studies were plagued by difficulties inherent in primate models, including length of time needed to create the lesions, and inter-animal variation in stability and severity of the MPTP lesion. The biggest limitation is in the availability of selective drugs which are orally available. While some inconsistent effects were observed in individual monkeys on parkinsonian signs, overall efficacy of *currently available* mGluR drugs in primate model of PD does not appear to be very promising. However continuing studies and further drug development will be essential to expand our understanding of the roles of mGluRs in basal ganglia function.

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APPENDICES

- I. Schematic diagram outlining the basal ganglia circuitry in normal and PD brains.
- II. Marino, Michael J., et al. Localization and Physiological Roles of Metabotropic Glutamate Receptors in the Indirect Pathway. In Movement Disorders, M. DeLong, A.M. Graybiel, S.T. Kitai, eds. (2000).
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A schematic representation of BG circuitry. The left panel represents normal transmission through the normal BG. The right panel represents the imbalanced transmission in the Parkinsonian BG. The solid arrows represent inhibitory projections, and the open arrows represent excitatory projections. The thickness of the arrow represents the relative activity of that particular projection. Thal, thalamus.

APPENDIX II

In Movement Disorders (2000)

LOCALIZATION AND PHYSIOLOGICAL ROLES OF METABOTROPIC GLUTAMATE RECEPTORS IN THE INDIRECT PATHWAY

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. The primary pathological change giving rise to the symptoms of Parkinson's disease is loss of dopaminergic neurons in the substantia nigra pars compacta that modulate the function of neurons in the striatum and other nuclei in the basal ganglia (BG) motor circuit (Fig. 1). Currently, the most effective pharmacological agents for treatment of PD include levodopa (L-DOPA), the immediate precursor of dopamine, and other drugs that replace the lost dopaminergic modulation of BG function¹. Unfortunately, dopamine replacement therapy ultimately fails in most patients due to loss of efficacy with progression of the disease and severe motor and psychiatric side effects². Because of this, a great deal of effort has been focused on developing new approaches for treatment of PD.

The primary input nucleus of the basal ganglia is the striatum (caudate, putamen, and nucleus accumbens), which receives dense innervation from the cortex and subcortical structures. The primary output nuclei of the basal ganglia are the substantia nigra pars reticulata (SNr) and the entopeduncular nucleus (EPN) which send GABAergic projections to the thalamus. The current model of cortical information flow through the basal ganglia states that the striatum projects to these output nuclei both directly, and indirectly through the globus pallidus and subthalamic nucleus (STN)^{3,4}. The direct pathway provides a GABAergic inhibition of the SNr/EPN, while the projection to globus pallidus relieves a GABAergic inhibition of STN, resulting in a glutamatergic excitation of SNr/EPN. A delicate balance between the inhibition of the output nuclei by the direct pathway, and excitation by the indirect pathway is believed to be crucial for control of movement, and any imbalance in this system underlies the pathophysiology of movement disorders.

Recent studies reveal that loss of nigro-striatal dopamine neurons results in a series

Figure 1. A model of how the Parkinson's-related loss of dopamine neurons in the SNc impacts information flow throught the basal ganglia. Note the increase of glutamatergic transmission at the STN-SNr synapse. Inhibitory connections are depicted by black arrows, excitatory transmission depicted by white arrows. Figure modified from reference 3.

of neurophysiological changes that lead to over activity of the indirect pathway, resulting in a pathological excitation of the STN. Increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neurons in the output nuclei. This glutamate-mediated over excitation of BG output ultimately produces the motor impairment characteristic of PD ⁵. Discovery of the pivotal role of increased activity in the indirect pathway in PD has led to a major focus on surgical approaches for treatment. For instance, lesions or high frequency stimulation of the STN provides a therapeutic benefit to PD patients⁶. In addition, pallidotomy, a surgical lesion of the GP, produces similar therapeutic effects by reversing the impact of increased activity of STN neurons^{7,8}. Development of these highly effective neurosurgical approaches provides a major advance in our understanding of the pathophysiology of PD. However, surgical approaches are not widely available to Parkinson's patients. Due to their invasive nature, high cost, and considerable expertise required, such treatment is reserved for patients that are refractory to dopamimetic therapy.

An alternative to surgical approaches to reducing the increased excitation of basal ganglia output nuclei in PD patients would be to employ pharmacological agents that counteract the effects of over activation of the STN neurons by reducing transmission through the indirect pathway. One approach would be to target metabotropic glutamate receptors (mGluRs). Eight mGluR subtypes have been cloned (designated mGluR1-mGluR8) from mammalian brain. These mGluRs are classified into three major groups based on sequence homologies, coupling to second messenger systems, and selectivities for various agonists. Group I mGluRs, which include mGluR1 and mGluR5, couple primarily to increases in phosphoinositide hydrolysis. Group II mGluRs (mGluR2 and mGluR3), and group III mGluRs (mGluR4, 6, 7, and 8) couple to inhibition of adenylyl cyclase. The mGluRs are widely distributed throughout the central nervous system and play important roles in regulating cell excitability and synaptic transmission (for review see ^{9,10}). One of the primary functions of the

mGluRs is a role as presynaptic receptors involved in reducing transmission at glutamatergic synapses. The mGluRs also serve as heteroreceptors involved in reducing GABA release at inhibitory synapses. Finally, postsynaptically localized mGluRs often play an important role in regulating neuronal excitability and in regulating currents through ionotropic glutamate receptors. If mGluRs play these roles in basal ganglia, particularly in the indirect pathway, members of this receptor family may provide an exciting new target for drugs that could be useful for the treatment of PD, as well as other disorders of BG function. In this chapter we will describe our current understanding of mGluR distribution and function in the indirect pathway. Unless otherwise noted, all results presented are from studies of rat basal ganglia.

THE STRIATO-PALLIDAL SYNAPSE

The indirect pathway arises from the striatal enkephalinergic medium aspiny neurons ^{11,12}. These GABAergic neurons project to cells in the GP, forming the first synapses in the indirect pathway. Striatal neurons express mRNA for group I, II and III mGluRs ¹³. Of these, the group III mGluRs, mGluR4 and mGluR7, have been localized to presynaptic striato-pallidal terminals using both confocal and electron microscopy ¹⁴⁻¹⁶. Neurons in the GP express mRNA for mGluR1 and 5 ¹³, and are immunoreactive for mGluR7 ^{15,16}. Postsynaptic localization has been demonstrated for mGluR1 ¹⁷, and mGluR7 ^{15,16}. In addition, mGluR5 has been localized to postsynaptic sites at primate striato-pallidal synapses¹⁸.

To date there have been no studies on the function of the mGluRs at the striato-pallidal synapse. However, the receptor localization raises some interesting possibilities. While the primary input to the pallidum is GABAergic, there is some sparse glutamatergic input from the STN ¹⁹. Therefore, activation of the STN could directly excite pallidal neurons by actions on postsynaptic ionotropic and metabotropic glutamate receptors, and disinhibit pallidal neurons by actions of the GP would in turn inhibit the STN. This inhibitory feedback loop may play a role in regulating the balance of activity through the indirect pathway under normal conditions. However, in the case of PD, the sparse glutamatergic input may be insufficient to maintain this feedback control. The potential therapeutic value of restoring balance at this site will be determined by future studies on the role of group III mGluRs in modulating transmission at this synapse.

THE PALLIDO-SUBTHALAMIC SYNAPSE

In contrast to the striato-pallidal synapse, relatively little is known about the distribution of mGluRs at the pallido-subthalamic synapse. The projection neurons of the GP express mRNA for mGluR1 and 5, and the glutamatergic projection neurons of the STN express mGluR1, 2, 3, and 5 mRNA¹³ Recently, the group I mGluRs have been postsynaptically localized to dendrites of STN neurons at both symmetric and asymmetric synapses ^{20,21}

Activation of group I mGluRs induces a robust depolarization of STN neurons ^{20,21}. Interestingly, this depolarization is blocked by the mGluR5-selective antagonist MPEP, but not by the mGluR1-selective antagonist CPCCOEt, indicating that only one of the group I mGluRs (mGluR5) localized at this synapse mediates the direct depolarization of these neurons. A role for the mGluR1 found at postsynaptic sites in the STN remains to be determined. In addition to directly depolarizing the STN neurons, group I mGluR activation also has been demonstrated to increase the frequency of STN burst firing ^{20,21, 22}. Since the switch from single spike activity to a burst-firing mode is one of the characteristics of parkinsonian states in

animal models²³⁻²⁵ and parkinsonian patients ^{26,27}, this effect may play a key role in the neuropathology of this disease.

THE SUBTHALAMO-NIGRAL SYNAPSE

Glutamatergic projections from the STN to the BG output nuclei constitute the final synapse in the indirect pathway. To date, the only study of mGluRs in the EPN has been an *in situ* study, and the results closely parallel findings in the SNr¹³. Therefore, we will focus on studies of the STN-SNr synapse. Neurons in the STN express mRNA for mGluR1, 2, 3, and 5, and the SNr GABAergic neurons express mRNA for mGluR1, 3, and 5¹³. Immunocytochemical studies have demonstrated presynaptic localization of mGluR2/3²⁸, and 7^{15,16} at asymmetric synapses in the SNr. The presence of mGluR2 is of particular interest because it exhibits a rather restricted distribution in the BG. In addition to the STN, the only other BG cells found to express mGluR2 are the striatal cholinergic interneurons ¹³. Therefore, compounds selective for mGluR2 would be expected to exhibit relatively few side effects. The group I mGluRs have been found postsynaptically localized at symmetric and asymmetric synapses in the SNr ^{17,29}.

Several recent studies have provided a great deal of information on the physiological roles mGluRs play in regulating the STN-SNr synapse. Both group II and group III receptors have been shown to inhibit glutamatergic transmission at this synapse ^{28,30}. In accord with the immunocytochemical studies, the pharmacology and physiology of this inhibition is consistent with actions on presynaptic mGluR2/3 and 7 ²⁸. Activation of group I mGluRs produces a robust direct depolarization of SNr GABAergic neurons ²⁹. This effect is blocked by the mGluR1-selective antagonist CPCCOEt, but not by the mGluR5-selective MPEP. Therefore, in contrast to the effect of group I mGluR agonists in the STN, this effect appears to be mediated solely by mGluR1. Interestingly, stimulation of glutamatergic afferents in the SNr at frequencies consistent with the normal firing rate of STN neurons induces an mGluR-mediated slow EPSP which is completely blocked by CPCCOEt ²⁹. This indicates that postsynaptic mGluR1 may play an important role in tonic regulation of basal ganglia output.

Since increased activity in the STN is believed to play a key role in the pathophysiology of PD ⁵, the STN-SNr synapse is a logical site to target pharmacological interventions. The findings that the group II mGluRs are effective at decreasing transmission at this synapse, and exhibit a somewhat restricted distribution, indicate that these receptors could provide an ideal target for the development of antiparkinsonian compounds. Consistent with this, recent studies have demonstrated that the systemic injection of the highly selective group II mGluR agonist LY354740 decreases haloperidol-induced muscle rigidity ³¹ and catalepsy ³⁰ in a rat model of PD.

METABOTROPIC GLUTAMATE RECEPTORS IN OTHER BASAL GANGLIA REGIONS

While this review has focused on the indirect pathway, it should be noted that mGluRs are expressed throughout the BG and have functional relevance at multiple sites. For example, input to the BG at the cortico-striatal synapse is modulated both presynaptically by group II and III mGluRs¹⁴⁻¹⁷ and postsynaptically by group I mGluRs^{17,32}. The main effect of the presynaptic mGluRs is to reduce the cortical input to the striatum ³³⁻³⁵. Activation of the postsynaptic group I mGluRs produce a direct excitation of the indirect pathway^{36,37}. Interestingly, mGluR5 has been found to exclusively colocalize with enkephalin in striatal

medium aspiny neurons ³² indicating that the selective activation of the indirect pathway may be mediated by this receptor.

The group III mGluRs mGluR4, and 7 have been localized to presynaptic symmetric striato-nigral terminals ¹⁴⁻¹⁶. Activation of group I and III mGluRs decreases inhibitory transmission in the SNr ³⁸, demonstrating that the mGluRs also play a role in modulating the direct pathway. In the case of the group I mGluRs, this is of particular interest since, as discussed above, mGluR1 has been demonstrated to directly activate SNr neurons. This direct excitation coupled with a group I-mediated disinhibition suggests that group I receptor activation could dramatically increase SNr output. Therefore, in addition to the relevance for PD, compounds selective for the group I mGluRs may hold therapeutic relevance for disorders involving alteration of activity through the direct pathway such as Huntington's disease, Tourette's syndrome, and epilepsy.

Finally, all three groups of mGluRs have been shown to modulate glutamatergic transmission in the substantia nigra pars compacta ³⁹. This finding is of particular interest since glutamate release in the SNc is hypothesised to play a role in the degeneration of the nigrostriatal dopamine system. While the source of the excitatory afferents regulated by mGluRs in SNc was not defined in these studies, it is likely that these EPSCs are mediated in part by activity at STN terminals. These data raise the exciting possibility that group II mGluR agonists have potential not only for reducing the symptoms of established PD, but could also slow progression of PD. Future studies will be needed to clearly define the role of increased STN activity in contributing to progression of the disorder and to rigorously define the mGluR subtypes involved in regulating transmission at STN-SNc synapses.

In summary, the mGluRs are expressed throughout the indirect pathway and selectively modulate synaptic transmission and cell excitability at each synapse in the pathway (table 1). Studies of this family of receptors not only provides insight to BG function, but holds promise for the development of therapeutic compounds for the treatment of movement disorders.

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Table 1. Summary of distribution and physiological effects of mGluRs in the indirect pathway. Numbers are indicated for mGluR subtypes detected at the mRNA or protein level. The mRNA columns refer to mRNA expression in the neurons of origin for the presynaptic terminals, and the target neurons for the postsynaptic terminals. See text for references.

PRESYNAPTIC LOCALIZATION AND EFFECTS

Synapse	mRNA (Presynaptic cells)	Protein (Presynaptic Terminal)	Physiological Effect
Striato-pallidal	1, 3, 4, 5	4, 7	?
Pallido-subthalamic	1, 5	?	?
Subthalamo-nigral	1, 2, 3, 5	2/3, 4, 7	Decrease Glutamate

POSTSYNAPTIC LOCALIZATION AND EFFECTS

Synapse	mRNA (Postsynaptic cells)	Protein (Postsynaptic Terminal)	Physiological Effect
Striato-pallidal	1, 5	1, 5, 7	?
Pallido-subthalamic	1, 2, 3, 5	1, 5	Direct Depolarization
Subthalamo-nigral	1, 3, 5	1, 5	Direct Depolarization

APPENDIX III

VALIDATION OF A FLOW ENZYME FLUORESCENCE ASSAY TO MEASURE GLUTAMATE USING MICRODIALYSIS IN AWAKE PRIMATES/

CONTINUOUS MEASRUMENT OF GLUTAMATE IN THE AWAKE PRIMATE, USING MICRODIALYSIS AND ENZYME FLUOROMETRIC DETECTION

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ABSTRACT

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We here describe a technique for on-line detection of glutamate in microdialysis samples in awake primates. The method is based on an enzymatic assay using fluorescence detection of glutamate, which in the past has been used combined to microdialysis in rats. We show that this method is relatively straightforward to use in primates, is highly specific and sensitive for glutamate (detecting approximately $0.5 \,\mu$ M/l glutamate). This methodology provides a continuous detection of glutamate and an improved time resolution to HPLC-based detection methods. These features make this technique ideal to use in microdialysis experiments to detect short-term changes in glutamate levels evoked by physiologic or pharmacologic manipulations in the primate brain.

INTRODUCTION

Microdialysis has been used extensively to measure glutamate in the central nervous system (CNS). In most cases, glutamate concentration in the microdialysates are currently being determined off-line, usually with high pressure liquid chromatography (HPLC) methods. These methods are highly specific and sensitive, but difficult, and expensive to set up and maintain. In addition, the time resolution of these methods is limited to 10-20 minutes in most cases (but see Lada et al., 1997). Also, the need to collect the samples and, in some cases to freeze them, can contribute to contamination problems.

Given these shortcomings of the HPLC-based methods, several groups have proposed alternative techniques, based on on-line analysis of glutamate using one of several enzymatic assays; as has been reviewed in detail by Obrenovitch and Zilkha, 2001). One of these methods uses the fluorescent properties of reduced nicotinamide adenine dinucleotide (NADH) formed by the metabolism of glutamate to alphaketoglutarate by glutamate dehydrogenase, in the presence of NAD⁺. The coupling of microdialysis to enzyme-fluorescence analysis was first described for lactate (Kuhr and Korf, 1988), and was later adapted to detect glutamate by Obrenovitch et al. (Obrenovitch et al., 1990). The combination of this method with microdialysis has been used extensively to monitor changes in brain glutamate levels in the rat (Dijk et al., 1994; Matsuda et al., 1998; Obrenovitch and Zilkha, 2001; Takita et al., 1997).

Measurement of glutamate levels <u>in vivo</u> in primates have significantly lagged behind similar measurements in rodents. Although there are numerous experimental questions that would benefit greatly from techniques to measure glutamate <u>in vivo</u> in
these animals, technical difficulties related to the methodology required to measure amino acids by HPLC, combined with the more general problem associated with conducting microdialysis in primates (see review by Bradberry, 2000) have resulted in the fact that this has only been done in very few studies (Enblad et al., 2001; Graham et al., 1989; Kling et al., 1993; Kodama et al., 2002; Sorkin et al., 1992; Yin et al., 1997).

The on-line fluorescence assay provides a valuable tool for detecting glutamate in the primate brain. The purpose of this report was to validate and troubleshoot this technique for microdialysis in the awake primate. We describe how we combined this method with a system that allows acutely placement of microdialysis probes in awake primates. As an example, we detail the application of this method to sampling glutamate levels in the basal ganglia of the primate.

MATERIALS AND METHODS

Animals

One juvenile male rhesus monkey (*macaca mulatta*, 4 kg) was used for the experiments described here. All procedures were performed in compliance with the *NIH Guide for the Care and Use of laboratory Animals* (1996) and were approved by the Emory University Animal Care and Use Committee. A few weeks before surgery, the animal was trained to sit in a primate chair and to be handled by the experimenter. Water and food were available <u>ad libitum</u>.

Surgical procedures

The animal was surgically prepared for the subsequent electrophysiologic recording and microdialysis sessions. Under aseptic conditions and isoflurane anesthesia,

a stainless steel recording chamber, 20 mm in diameter, was stereotactically positioned over a trephine hole in the skull, and held in place with dental acrylic. The chamber was aimed at the putamen (A=12, L=10), at an angle of 50° from the vertical in the coronal plane. Metal head holders were also embedded into the acrylic cap to serve as bolts to stabilize the head of the monkey during the recording and microdialysis procedures.

Mapping procedures

The monkey stayed awake during all mapping and microdialysis sessions. The animal was seated in a primate chair with its head restrained, but free to move its body and limbs. Initial electrophysiologic mapping served to outline the dorso-ventral, anterio-posterior and medio-lateral borders of the putamen. Extracellular recording provided a reliable map of the structure, thus maximizing the accuracy of placement of the microdialysis probes.

A microdrive (MO-95B, Narishige, Tokyo, Japan) was used to lower tungsten microelectrodes (impedance 0.5-1.0 Mohms, FHC, Memphis, TN) into the brain through a 20-ga guide tube that penetrated the dura and protected the electrodes from damage while passing the dura. The signal was amplified (DAM-80 amplifier, WPI, Sarasota, FL; MDA-2 amplifier, BAK electronics, Germantown, MD), filtered (400 Hz-6 k Hz, model 3700, Krohn-Hite), displayed on a digital oscilloscope (DL1540, Yokogawa, Tokyo, Japan), and made audible via an audio amplifier.

Multiple electrode penetrations into the putamen were performed, and individual neurons were identified by their discharge characteristics, and by their relationship to neighboring identifiable structures, such as cortex and external globus pallidus (Crutcher and Alexander, 1990; DeLong, 1971)

Microdialysis procedure

Microdialysis probes

The microdialysis probes were custom made by CMA Microdialysis (North Chelmsford, MA). The probes had a concentric design, and carried a cuprophane membrane (6,000 MW cut-off) with an exposed length of 2 mm and an outside diameter of 0.24 mm. The inner cannula was constructed of fused silica coated with polymide tubing, while the outer shaft was stainless steel tubing with a 0.38 mm diameter. The probes were 135 mm long, so that they could be mounted in the microdrive instead of the microelectrodes mentioned above, and provide full access to the basal ganglia nuclei. Owing to their size, these probes have a considerable dead volume (9.64 1), which had to be taken into account when calculating the transport time for the dialysate.

We modified these commercial probes before using them in our system (Fig. 1). A tube system, consisting of concentric 19- and 23-ga stainless steel tubes (8 and10 mm long respectively) was glued to the end of the probe s standard metal shaft, next to the plastic piece where inlet and outlet tubes enter the probe. In addition, a sliding 23-ga 15 mm tube piece was placed around the probe itself, to help protect the membrane during insertion of the microdialysis probe into a guide cannula (Fig. 1B). During insertion, this protection tube engaged with the upper end of the guide cannula, and was pushed upwards along the shaft of the microdialysis system (Fig. 1C). After placement, the probe was secured in place with a small acrylic c-shaped clamp (Fig. 1D).

As manufactured, the probes are shipped in glycerol solution. Prior to use, they were rinsed by first dipping them in 70% ethanol for 5 minutes, as recommended by the manufacturer, followed by flushing with artificial CSF (aCSF, composition below) at 4

 μ l/min. This procedure also serves to sterilize the membrane. The ethanol was removed from the outside of the membrane by dipping the probe in aCSF for an additional 5 min. At the end of this procedure, the tip of the membrane was inspected to verify that it was free of air bubbles.

In order to re-use the probes, at the end of each experiment the probe was rinsed with distilled water. In our experience, the microdialysis probes can be re-used at least three times. The use is limited by mechanical failure of the probe, and by gradual failure of the membrane to recover glutamate. While the former is obvious on inspection of the probe, the latter was tested by performing calibration routines at the end and start of each experiment.

Microdialysis procedure

Before placing the probe into the brain, the probe was calibrated by exposing it for 10 min to 50 μ M/l glutamate in aCSF. The same calibration routine was repeated at the end of each experiment to detect changes in the recovery rate of the probe. Experiments were discarded if the difference between the first and the second calibration pulse was greater than 10%. When a microdialysis probe was re-used, it was verified that the response to 50 μ mol/l of glutamate did not change more than 10% from the previous calibration.

To place the microdialysis probe in the striatum, the microdrive was mounted on the recording chamber and the guide cannula was lowered into the brain, through a 20-ga tube that punctured the dura. The guide cannula was targeted 1 mm above the final location of the probe (as determined by the previous mapping sessions). For placement of the guide tube into the brain, a metal stylet (27-ga, same length as guide cannula) was

inserted into the tube to prevent dura or brain tissue from entering the cannula. Once the guide cannula was in place, the stylet was removed and the microdialysis probe inserted instead (protected by the metal 23-ga tube as mentioned above), and secured in place using the acrylic c-shaped clamp (see Fig. 1D). Thus, the probe sat tightly in the guide cannula without turning or sliding. Microdialysis penetrations were spaced at least 1 mm from each other, to avoid extensive tissue damage.

The probe was perfused with aCSF (composition in mmol/l: 143 NaCl, 2.8 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 1 Na₂HPO₄), at a 2 μ l/min flow rate, using a microinjection pump (CMA/102). A liquid switch (CMA/110) was used to enable switching of the perfusion fluids without introduction of air into the system.

All pharmacologic manipulations reported here were accomplished by changing the composition of the perfusion medium. Depolarization of the tissue surrounding the microdialysis system was achieved by perfusing the microdialysis system briefly with high potassium solution (aCSF containing 80 mmol/l KCl and 65.8 mmol/l NaCl.). In other experiments, the potassium channel blocker 4-aminopyridine (4-AP, 1mmol/l) was also used to induce depolarization. The calcium dependency of basal and stimulated glutamate levels was tested by perfusing the membrane with aCSF containing 0 mmol/l $CaCl_2$, 2.2 mmol/l MgCl₂, in the presence of the calcium chelator ethylene glycolbis(beta-aminoethyl ether)-n,n,n ,n -tetraacetic acid (EGTA,1 mmol/l). Before being loaded in the syringes, all solutions to be perfused through the microdialysis probe were filtered using a nylon membrane with a 0.2 μ m pore size.

Enzyme fluorometric analysis of glutamate

Glutamate was determined by on-line fluorometric detection of NADH, produced by the reaction of glutamate and NAD⁺ to alpha-ketoglutarate and NADH catalyzed by glutamate dehydrogenase (Graham and Aprison, 1966). The reaction requires ADP, and is carried out in the presence of hydrazine which removes the reaction end product (_ketoglutarate) from the solution, effectively resulting in quantitative transformation of glutamate.

The reactant solution consisted of 5.4 U/ml glutamate dehydrogenase (EC 1.4.1.3.), from bovine liver (Roche Diagnostics, Indianapolis, IN), 0.34 mmol/l nicotinamide adenine dinucleotide (NAD⁺, Sigma-Aldrich, St Louis, MO), 1.5 mmol/l adenosine diphosphate (ADP, Sigma-Aldrich), and 0.29% hydrazine hydrate (Sigma-Aldrich) in Tris buffer (100 mmol/l) The pH of the reactant solution was adjusted to 8.5.

The reactant solution was filtered and loaded in gastight syringes (Bioanalytical Systems, West Lafayette, IN), and the flow rate adjusted at 6µl/min using a microinjection pump (CMA). The reactant solution was mixed with the dialysate emerging from the microdialysis probe using a three-way metal tubing connector (23-ga, Small Parts Inc., Miami Lakes, FL). The 3:1 reactant/dialysate ratio used in this report is lower than that described by Obrenovitch et al (1990, 20:1). This reduces the dilution of the dialysate by the reactant, and enhances the sensitivity of the assay.

The enzymatic reaction occurred in a tubing assembly composed of 938 mm of FEP tubing (ID 0.12 mm, CMA) plus 283 mm of Teflon tubing (ID 0.3 mm, Small Parts). This combination of tubing was used to achieve a convenient distance of tubing without an excessive volume. The dead volume of this tubing assembly (around 30 μ l) permitted

an incubation time of 5.5 min. (from the point of mixing to the inlet of the spectrofluorometer). All connections were made using Teflon tubing adapters (CMA).

This setup allowed for continuous flow of the microdialysate to the fluorescent detector. NADH in the dyalisate was detected with a fluorescence detector (RF-10AXL, Shimadzu Scientific Instruments Inc., Tokyo, Japan) with a 12 μ l flow cell, at 340-450 nM excitation-emission wavelengths. Different sensitivity/gain combinations were tried in order to optimize the signal/noise ratio. The final settings (sensitivity=1, gain=1) resulted in an overall gain of 1024x (in a 1-16384x range).

The fluorescence detector generates a DC voltage which is proportional to the measured fluorescence. The dynamic range of the detector is set so that a fluorescence of zero reflects a certain baseline fluorescence. Between this pedestal and the upper limit of its dynamic range, fluorescence levels are linearly recorded. Absolute measurements of glutamate levels can be gleaned from calibration curves using different concentrations of glutamate.

The DC voltage generated by the fluorometric detector was acquired using a analogical-digital interface, and continuously recorded to computer disk at a sampling rate of 10 Hz using LabView Software (National Instruments, Austin, TX).

RESULTS

1. <u>In vitro</u> testing of the enzymatic assay

1.1. Background fluorescence

Compared to bi-distilled water, the reactant-aCSF mixture (free of glutamate) produced a fluorescent signal of around 0.7 V. This background fluorescence was omitted by re-zeroing the signal after it was stable.

All compounds that we intended to use were tested <u>in vitro</u> for their ability to produce background fluorescence. If a compound gave a fluorescent signal equal or larger than the signal resulting from exposure of the probe to 5 μ mol/l glutamate (approximately 0.02 volts, Fig 2), it was not used in our experiments. This was true for only a small number of compounds. As an example, the GABA_B antagonist baclofen (not used in the studies reported in detail here) when perfused through the probe produced a considerable fluorescent signal (around 0.1 V). This was, however, an uncommon problem; most of the compounds tested so far showed no fluorescent signal on their own. *1.2. Dose-dependence of fluorescence detection of concentrations of glutamate*

To evaluate the relationship between glutamate concentrations and assay response and to find the detection limit, increasing concentrations of glutamate were injected into the fluorometric detector. To do this, each glutamate solution was loaded in a syringe and mixed with the reactant solution, using the same tubing system described for the microdialysis experiments, to maintain the same incubation time. The lowest concentration which gave a fluorescent signal which could be reliably distinguished from the noise was 0.5 µM of glutamate (Fig. 2A).

The dose response curve was repeated, but instead of injecting the glutamate solutions in the detector, a microdialysis probe was exposed to increasing concentrations of glutamate, which also resulted in a dose dependent response (Fig. 2B). The response was linear throughout the concentration range tested (linear regression yielded a regression coefficient of 0.9986, see figure 2C).

For each of the subsequent experiments, calibration of probes was done by exposing the probe to 50 μ mol/l glutamate in aCSF (an example is shown in Fig 3A). Pooled data from seven probes showed that the average fluorescent response to the calibration pulse was 0.45 +/- 0.02 V (mean +/- S.E.M), and the average time to reach a stable response was 361.9 +/- 13 s. (mean +/- S.E.M.). In our experiments there was no relationship between the time to reach a stable response and the glutamate concentration (i.e., the same lag applied to low and to high glutamate concentrations). In our experiments there was no relationship between the time to reach a stable response and the glutamate concentration (i.e., the same lag applied to low and to high glutamate concentrations).

1.3. Specificity testing

The specificity of the assay was tested by exposing the probe to solutions of various chemically closely related amino acids, all used at 50 μ mol/l.

The following amino acids were tested: GABA, aspartate, glutamine, ascorbate, taurine, valine, alanine, and D-glutamate. In these tests, only glutamine gave a significant signal, around 1/3 of that given by glutamate (not shown). Interestingly, this may not be due to lack of specificity of the glutamate dehydrogenase, (see, e.g., Smith et al., 1975), but the result of low-level bacterial contamination within the microdialysis system,

resulting in the rapid transformation of glutamine to glutamate (White, 1995). Indicative for this problem is the fact that the glutamine responses were inconstant, and were abolished after cleaning the flow detector cell, and replacing the tubing and probes. The glutamine response was not stimulated by concomitant exposure to high potassium.

2. <u>In vivo</u> testing of the fluorometric assay

2.1. Response after placing probe in brain

Immediately after placing the microdialysis probes into the putamen of the awake monkey, a rapid increase in fluorescence was recorded (Fig. 3B). This initial peak was, in all experiments, out of range for the detection system. However, after several minutes the signal started to decrease in an exponential fashion. In an initial experiment, the glutamate level was monitored for four hours without additional experimental manipulations. As shown in figure 3B, it was confirmed that a steady state was reached 1 hr after probe insertion.

Using the calibration pulse as a yard stick, the basal glutamate levels were calculated as the level of fluorescence at the steady state compared to the background fluorescence observed before placing the probe in the brain. The average glutamate basal level thus measured was 28.74 +/- 2.73 μ M (mean of seven microdialysis penetrations +/- S.E.M.).

2.2. K⁺ evoked glutamate release in the striatum

A common test for the neuronal origin of chemicals sampled through microdialysis is to measure changes in the levels of the putative transmitter after exposure to depolarizing concentrations of potassium. Using this approach, we measured changes in glutamate levels in the striatum by reverse dialysis of high potassium (80 mmol/l). To this end, 80 min after probe insertion, regular aCSF was replaced by aCSF containing 80 mmol/l of

potassium. The glutamate level, as measured by the intensity of fluorescence, increased reliably by an average of 0.136 V, which corresponded to 24.81 µmol/l of glutamate. Subsequent stimulation pulses produced also increases in the signal, although after the first pulse the amplitude of the responses decreased with each additional pulse (see Fig. 4A). Since the calibration pulse was the same at the end of the experiment, the decrease on potassium-evoked glutamate response cannot be attributed to loss of recovery, but likely represents a true reduction in tissue glutamate release

2.3. Calcium dependence of potassium evoked glutamate release

As another test of the neuronal origin of the measured glutamate levels, the calcium dependency of the potassium-evoked glutamate release in the striatum was assessed. Ten minutes before the potassium stimulation, the regular aCSF was switched to calcium-free aCSF with 1 mmol/l EGTA. Under these conditions, the high potassium-evoked release of glutamate was importantly decreased (figure 4B). Calcium-free aCSF, on its own, produced a transient small increase in fluorescence, likely reflecting a small amount of glutamate release. Calcium-free aCSF did not produce a fluorescence signal in vitro.

2.4. 4-AP evoked release of glutamate

As an alternative means of discerning the neuronal origin of the glutamate release, the blocker of voltage-gated potassium channels, 4-aminopyridine (4-AP) was used. The administration of 1 mmol/l of 4-AP through reverse dialysis produced repeatable glutamate responses (not shown).

DISCUSSION

These results show that glutamate can be reliably detected in the striatum of awake primates using microdialysis coupled to an online enzyme-fluorescence assay. The identity of glutamate was verified by <u>in vitro</u> test of other amino acids. Glutamate levels are increased in a calcium-dependent manner by tissue depolarization with high potassium or 4-AP, indicative of a possible neuronal source of the measured glutamate levels. The use of a microdrive to position the microdialysis probes in the brain allows for multiple microdialysis penetrations at different locations in the same animal. The obvious advantage of this approach is that issues related to topographic differences in biochemical constituents can be addressed in the same animal, that the dialyzed tissue can be chosen to be undamaged, and that animal use is optimized.

Our validation procedure was undertaken to assess that the signal detected in our experiments corresponds indeed to glutamate. It has been shown that the glutamate dehydrogenase itself does not evoke an increase in fluorescence (Takita et al., 1997). Our results suggest that other, closely related, amino acids do not change the background fluorescence. If microbial contamination is present in the tubing system, glutamate can be formed from glutamine, which can be an important source of contamination on biological conditions, since glutamine is present at high concentrations in the extracellular fluid. Using an aseptic technique seems to be sufficient to keep the system free of microbial contamination, at least on our short-term studies. However, others have reported that sterilization of probes is necessary if they are going to be implanted for periods longer than 24 hr (Zhou et al., 2002).

This assay provides a linear relationship of concentration of glutamate vs. intensity of fluorescence, at least in the range of concentrations tested (present report and Takita et al., 1997). The intensity of fluorescence can, thus, be considered a direct indication of the glutamate concentration in the microdialysate.

Both <u>in vivo</u> and <u>in vitro</u>, the fluorescence reached a stable peak after about 6 min. Matsuda et al (2000) reported that the time to reach 90% of the maximum response was close to 2 min. The relatively slow rise of the glutamate signal even after sudden exposure reflects most likely the time it takes for the mixture of reactant and dialysate to completely fill the flow cell (12μ L). The low-pass filter characteristics of the assay are a significant limitation, because rapid changes in glutamate levels, as may be encountered in physiologic experiments can clearly not be reliably detected. However, as we show here, the system is capable of detecting pharmacological changes induced by stimulation with potassium or 4-AP. Additional preliminary observations indicate that the technique can also monitor changes in glutamate levels evoked by electrically stimulating nuclei that give rise to glutamate projections to the area undergoing microdialysis.

When the probe was placed in the striatum, the fluorescence intensity exceeded the linear range of this method. It has been proposed that this abundant release of neurotransmitter is produced by local brain injury (Benveniste et al., 1989). Despite this major elevation in glutamate levels, in our experiments a steady state was reached after about 1 hour. The fact that only one hour is necessary to achieve stable glutamate levels, and that this process can be monitored in real time, is clearly of importance as the total experiment time needs to be limited as far as possible particularly when the animal remains awake.

We found that in the monkey striatum, glutamate basal levels were 28.74 +/- 2.73 μ M/L. To the best of our knowledge, glutamate levels have not been previously reported for the striatum in the non-human primate. Values in the monkey globus pallidus were reported to be 0.05-0.5 μ M/L (Graham et al., 1989), and in the rat, glutamate basal levels are usually found to be around 1 to 5 μ M/L, either measured by HPLC (Rawls and McGinty, 1997 and references therein), or using the on-line fluorescence detection method (Obrenovitch et al., 1993; Obrenovitch et al., 1997; Takita et al., 1997). The higher values found in the primate may indicate differences in the density or activity of glutamate afferents between species.

Advantages of the enzyme-fluorescence assay

The advantages and limitations of this online enzymatic assay to measure glutamate have been discussed previously by Obrenovitch (2001). Probably the most significant advantage of the method is that changes in the glutamate levels can be monitored within a few minutes of their occurrence in the brain, eliminating problems with storage and freezing of dialysate samples for later analysis. In contrast to standard HPLC methods, the fluorescence detection assay is relatively easy to implement and low in cost, and the routine maintenance is not time consuming. If the microdialysis technique is already established, the addition of online enzyme fluorescence can be easily achieved.

Limitations of the enzyme-fluorescence assay

One problem with the fluorescence based assay is that the detection limit of the assay is lower than that of HPLC methods. This is in part due to the dilution of the dialysate by the reactant. In our experience, the optimal signal-to-background ration was obtained by

using a 1:3 ratio between dialysate and reactant, i.e. using an injection flow rate of 6 μ l/min for the reactant and 2 1 for the dialysate. The fluorescent signal is also sensitive to mechanical disturbances. For example, turning pumps on or off may introduce artifacts, and small ripple artifacts originate from the periodic error in the pump gear system during normal operation of the pump. Such artifacts can be limited by using high-quality pumps, liquid switches and tubing, and by keeping all perfusion and reactant solutions free of air bubbles. In general, the mechanical interference seen is small enough to not interfere with the glutamate detection. The specificity of the fluorescent signal for glutamate (and of any other method of glutamate detection) can also be compromised by bacterial contamination, resulting in conversion of glutamine to glutamate (see above). This, of course, can be eliminated by using aseptic techniques, and frequent changes of tubing, connectors and probes. The specificity of the assay can (and should) be easily monitored by exposing the probe to high levels of glutamine.

Neuronal origin of glutamate measured by microdialysis

Although the origin of glutamate as measured through microdialysis techniques remains controversial (Nedergaard et al., 2002; Obrenovitch, 1998; Timmerman and Westerink, 1997), our studies provide some evidence in favor of the view that the glutamate measured with our system could originate, at least in part, from neuronal release. In agreement with other studies (Paulsen and Fonnum, 1989) Welsch-Kunze et al., 1993) (Bakkelund et al., 1993) (Rowley et al., 1995; Ueda et al., 2000) (Obrenovitch et al., 1993; Swanson et al., 2001), we found that glutamate levels were increased by tissue stimulation with potassium, and that the potassium-evoked glutamate release was calcium dependent. Interestingly, there was a small increase in glutamate level when the

dialysis probe was perfused with calcium-free solution (see also Miele et al., 1996)). A putative explanation for this phenomenon is that the decrease in calcium inhibits the release of other neurotransmitters (e.g. dopamine), which presynaptically modulate the release of glutamate (Yamamoto and Davy, 1992)

In addition, we observed increased glutamate levels in response to AP-4 infusion in the monkey striatum, which may also serve as evidence in favor of neuronal release of glutamate in our preparation. Finally, in a few preliminary experiments we have also demonstrated that glutamate levels in the pallidum increase upon electrical stimulation of the subthalamic nucleus (unpublished), demonstrating further that the system described here is capable of measuring neuronal glutamate release.

In conclusion, the method described here appears suitable to monitor changes in the neuronal release of glutamate in awake primates. This technique should be usable for pharmacologic and physiologic studies, particularly investigating presynaptic modulation of glutamate release in these animals. Fig. 1: Probe and probe assembly. (A) Microdialysis probe, as manufactured byCMA Microdialysis. (B) Tubing adaptors added to fit the probe in our system (C)Insertion of the microdialysis probe in the guide tube. This is done once the guide tube ispositioned in the brain, and the sliding piece is placed over the membrane to protect it.(D) When both the guide cannula and probe are placed in location inside the brain, anacrylic c-clamp with two screws is used to fix the assembly in place.

Fig 2. The fluorescence intensity corresponds to the concentration of glutamate. (A) Increasing doses of glutamate (as indicated by the arrows) were mixed with the reactant solution and injected into the fluorescent detector. (B) A microdialysis probe was dipped consecutively in increasing concentrations of glutamate. (C) A linear relationship (r=0.9986) was found between concentrations of glutamate and fluorescence intensity

Fig 3: Glutamate levels measured <u>in vitro</u> to calibrate the probe and insertion of the probe into the striatum. (A) Representative response, resulting from exposure of the microdialysis probe to a solution containing 50 µmol/l of glutamate (black line on bottom indicates insertion of the probe in the glutamate solution). The time for the peak to reach a steady state is indicated by dashed line. (B)After calibration, the probe was inserted in the striatum of the monkey. The arrow marks the time when the probe was inserted in the brain. Note that a stable baseline is reached in less than one hour after insertion.

Fig 4: Potassium evoked changes in glutamate levels in the striatum of the awake primate. (A) Repeated potassium stimulations (80 mmol/l) substantially increase the levels of glutamate in the striatum. For the purpose of this figure, the fluorescent signal was converted to glutamate concentration based on the calibration of the microdialysis probe in a 50 mol/l glutamate solution. (B) Pre-perfusion of the microdialysis probe with

free calcium medium (containing EGTA) diminished the subsequent potassium-evoked release of glutamate. The black line indicates perfusion with 80 mmol/l of potassium, and the gray line indicates perfusion with free calcium and 1 mM/l EGTA.

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A

B





Glu concentration (µM)





APPENDIX IV

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METABOTROPIC GLUTAMATE RECEPTORS IN THE GLOBUS PALLIDUS

Olga Poisik¹, Yoland Smith¹, and P. Jeffrey Conn²

1. INTRODUCTION

Classically presented as a relay nucleus of the indirect pathway, the external globus pallidus (GPe in primates or GP in rodents) integrates information transmitted along several inputs. The inhibitory GABAergic innervation from the striatum (Str) and the excitatory glutamatergic afferents from the subthalamic nucleus (STN) primarily control the activity of GP. GP neurons integrate the two disparate inputs and the relative weight of each input determines the information flow out of the nucleus. The objective of this chapter is to discuss the contribution of the glutamatergic transmission towards the output of the GP, with the emphasis on the role of metabotropic glutamate receptors in modulating the excitability of GP neurons.

2. SYNAPTIC TRANSMISSION IN THE GP

The STN provides the second most abundant innervation of the GP, while inputs from the Str remain the most profuse. The STN is the only glutamatergic structure within the basal ganglia and it sends efferents to all nuclei of the basal ganglia circuit. The activity of STN is predominantly controlled by the excitatory inputs from the cerebral cortex and the inhibitory afferents from the GP. Neurons in the STN also receive inputs

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from the substantia nigra, the centromedian-parafascicular nucleus of the thalamus, the dorsal raphe, and the pedunculopontine tegmental nucleus (Parent and Hazrati, 1995), (Smith et al., 1998).

It has long been recognized that GP and STN are reciprocally linked according to a very tight pattern of synaptic connectivity (Shink et al., 1996). In a normal state, the discharge patterns of the GP and the STN do not exhibit any correlation (Bergman et al., 1998). The synchronization of the GP-STN output, however, can be achieved by general anesthesia (Magill et al., 2000). In this paradigm, the rhythmic input originating from the cerebral cortex is still required for phase locking the GP-STN output in vivo.

Cortical information predominantly reaches the GP via two projection tracts, the cortico-striato-pallidal tract and the cortico-subthalamo-pallidal tract. Furthermore, there is evidence for a direct projection to the GP from the cerebral cortex (Naito and Kita, 1994). These inputs are excitatory and display topographic organization. However, the contribution of the direct cortical excitation towards the output of the nucleus remains unclear. It is also not clear how cortico-pallidal synapses are organized in the GP compared to the innervation pattern of excitatory STN fibers.

In primates, axon collaterals from the STN permeate GPe and GPi in the form of elongated bands. Functionally related STN neurons project to a restricted set of pallidal neurons (Shink et al., 1996). In primates and rats, subthalamopallidal fibers terminate on both the cell body and dendrites of GP neurons (Smith et al., 1998). These terminals comprise about 10% of all inputs to the GP in both species (Shink and Smith, 1995), (Falls et al., 1983), (Smith et al., 1998).

On the other hand, the striatopallidal fibers comprise over 80% of all terminals in rat and monkey pallidum (Falls et al., 1983), (Shink and Smith, 1995). Striatal projections to GP are highly specific and show a high degree of arborization within the nucleus (Smith et al., 1998).

Like STN, the activity of the dorsal Str is primarily controlled by glutamatergic inputs from the cerebral cortex. Fibers from the dorsal Str and STN terminate on the same neurons in GPe and GPi. (Hazrati and Parent, 1992). The striatal afferents intertwine with the dendrites of individual GPe neurons, while subthalamic inputs contact both dendrites and perikarya. It remains to be established whether striatal and subthalamic fibers contacting the same cells in the GPe are functionally related.

Over the past ten years it has become well established that the GP serves as an integrator of two opposite signals that originate in the cerebral cortex (Bolam et al., 2000). Indeed, stimulation of the somatosensory cortex in anesthetized rats first elicits an excitatory response in the GP that is presumably transmitted via STN. This excitation is followed by the inhibition from the striatum (Kita et al., 1992). Therefore, it appears that information from the cortex would travel faster along the cortico-subthalamo-pallidal tract than along the cortico-striato-pallidal projection. The initial excitation may alter the response of GP neurons to inhibitory inputs from Str. This observation emphasizes the importance of the cortico-subthalamic projection in the functional circuitry of the basal ganglia.

Another recent finding suggests that the balance between excitatory and inhibitory inputs in the GP is shifted to favor excitation. Type I and type II Na⁺ channels are associated post-synaptically with glutamatergic synapses in the rat GP (Hanson J.E. et al., 2000). Enrichment of these channels at asymmetric synapses can boost the neuronal response to glutamate.

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Although fibers from Str and STN comprise the majority of terminals in GP, other inputs to this nucleus should not be ignored. The GP also receives glutamatergic inputs from the thalamus, dopaminergic inputs from SNc/VTA, serotonergic inputs from the raphe, and cholinergic/glutamatergic inputs from the PPN (Smith et al., 1998), (Pasik et al., 1984). These fibers collectively comprise about 5% of all terminals in the monkey GPe (Shink and Smith, 1995). The synaptic organization of most of these inputs has been poorly characterized (Clarke et al., 1996). There is evidence that dopaminergic inputs may modulate GABAergic transmission in the rat GP. Activation of D2 dopamine receptors, which are most abundantly expressed on striatal afferents in the GP, inhibits locally evoked IPSCs (Smith and Kieval, 2000), (Cooper et al., 2001).

3. METABOTROPIC GLUTAMATE RECEPTORS IN THE GP

Activation of metabotropic glutamate receptors (mGluRs) in the GP may modulate excitatory and inhibitory transmission. The mGluRs are G-protein coupled receptors that can extensively modulate the excitability of neurons throughout the central nervous system (Anwyl, 1999). These receptors have been subdivided into three groups based on their amino acid sequence homology. Eight subtypes have been cloned thus far, many of which possess a number of splice variants (Conn, 1994), (Pin and Duvoisin, 1995). For example, mGluR1, member of the group I mGluR family, has 5 known splice variants that are termed mGluR1a through mGluR1e.

Members of groups I and III of the mGluR family are richly expressed in the GP. Group I mGluRs, mGluR1a and mGluR5, localize postsynaptically to all GP neurons (Testa et al., 1998), (Hanson and Smith, 1999). In the rat, mGluR1a immunorectivity is predominantly associated with dendrites (Testa et al., 1998). In the primate, mGluR1a and 5 localize to perikarya and dendrites where both receptors are primarily extrasynaptic (Hanson and Smith, 1999). Group I immunoreactivity is also found peri-synaptically to the postsynaptic specializations of STN afferents. Interestingly, a significant percentage of group I mGluRs localize to symmetric synapses that are formed by the GABAergic terminals from the striatum. The exact functional role of these mGluRs at GABAergic synapses and their source of activation are still unknown.

MGluR4a and 7a, two members of the group III mGluRs, are also abundant in the GP. Both receptors are likely to be expressed presynaptically, although mGluR7a is also found postsynaptically (Kosinski et al., 1999), (Bradley et al., 1999). MGluR4a is thought to function as a heteroreceptor at GABAergic synapses. Lesion of striatopallidal fibers by quinolinic acid, indeed, reduces mGluR4a immunoreactivity in the rat GP (Bradley et al., 1999).

Very little is known about functions of mGluRs in the GP. However, extensive data on mGluR functions have been collected in other systems throughout the CNS. Activation of group I mGluRs, which are very abundant in the GP, induces slow and long lasting stimulatory effects in many neuronal populations in CNS. Most notably, in the hippocampus, stimulation of post-synaptic group I mGluRs inhibits I_{AHP} , I_M , and I_K^+ (leak) (Anwyl, 1999). Inactivation of these currents promotes neuronal excitability. Activation of group I mGluRs has also been demonstrated to potentiate NMDA currents, which is thought to facilitate long-term pontentiation (LTP) (Otani et al., 1993).

The two pre-synaptic mGluRs in the GP, mGluR4a and mGluR7a, have been shown to potently depress synaptic transmission in other systems. For example, activation of group III mGluRs depresses IPSCs in the SNr (Wittmann M, 2001). This evidence reveals that group III mGluRs can commonly function as heteroreceptors at GABAergic synapses in basal ganglia structures. It is, therefore, very likely that activation of group III mGluRs in the GP reduces GABA release at striato-pallidal synapses, thereby increasing post-synaptic inhibitory influence of GP neurons in the STN. Decreasing activity of STN neurons may be beneficial in Parkinson's disease (PD), since the hyperactivity of the subthalamofugal projections is a cardinal feature of PD pathophysiology. Therefore, agonists that are selective for group III mGluRs should be considered as a potential therapeutic strategy in PD.

Thus, activation of mGluRs in the GP can profoundly affect the output of this nucleus, which projects to basal ganglia and non-basal ganglia structures. Single cell tracer injections reveal that the axons of GP neurons are highly collateralized and terminate in a number of target structures. The main projection site of GP neurons is the STN, but additional efferents to Str, GPi/SNR, PPN, and the reticular nucleus of the thalamus have also been disclosed (Smith et al., 1998), (Parent and Hazrati, 1995). In addition, there is evidence that GP neurons provide intrinsic axon collaterals in rats. (Kita and Kitai, 1994), (Bevan et al., 1998).

4. NEURONAL POPULATIONS IN THE GP

GPe neurons are GABAergic and inhibit their target structures. In vivo recordings in rats and primates reveal that about 70% of GP neurons fire spontaneously (Kita and Kitai, 1991), (Mitchell et al., 1987). A number of studies focused on characterizing these neuronal populations in vitro. At least three neuronal populations have been described thus far in rodents based on electrophysiological and morphological criteria. Results of



Figure 1. A. *i*. Type I GP neuron, *ii*. type II GP neuron. Cells were held at -70 mV and stepped to -109 mV. Arrow indicates presence of time and voltage-dependent inward rectifying current I_h. **B**. Cells were held at around -60 mV and stepped to different membrane potentials using shown current injection paradigm. Arrow indicates a sag in the membrane potential, which corresponds to the presence of I_h.

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these studies are often inconsistent due to differences in technique, age of animals, and animal species used. However, a set of criteria has been worked out to describe different neuronal populations in the GP.

At least two subpopulations are thought to be projection neurons. Type A or type II neurons are characterized by a relatively high input resistance, weak spike frequency adaptation, and the presence of time and voltage-dependent inward current I_h (Cooper and Stanford, 2000), (Nambu and Llina, 1994). These neurons are often spontaneously active. On the other hand, type C neurons (Cooper and Stanford, 2000), which are much less abundant, are characterized by the absence of I_h and presence of a ramp-like depolarization during a depolarizing step. These cells are always quiescent at rest.

5. RESULTS

In our studies, we utilize whole cell patch clamping technique to record from GP neurons in brain slices of juvenile rats (15-18 days old). So far, we have recorded from over 200 GP neurons. In our preparation, we have encountered two neuronal sub-types based on published electrophysiological criteria described above. We have termed these neuronal populations type I and type II. The type I GP neurons are characterized by a lower input resistance ($405\pm20~M\Omega$), ramp depolarization during the depolarizing step, and lack of time and voltage-dependent inwardly rectifying current I_h. On the other hand, the type II neurons are characterized by higher input resistance ($813\pm159~M\Omega$), lack of ramp depolarization during the depolarizing step, and presence of time and voltage-dependent inwardly rectifying current I_h (Figure 1). About 80% of type II neurons are active at rest while type I neurons are always silent. Our type II neuronal population



Figure 2. A. i. Examples of type I GP neurons, or ii. Type II GP neurons. Scale bar: 25 µm.
B. Relative position of the traced neuron in GP. i. type I GP neuron, ii. type II GP neuron. The dorsal (D) and posterior (P) orientations of the slice are indicated. Scale bar: 100 µm.

Patch solution contained biocytin at 0.5 mg/ml. Biocytin was allowed to diffuse passively into the cell during recording. After recording slices were fixed with 10% PFA. Filled neurons were visualized using streptavidin-HRP/DAB method. Str-striatum, GP-globus pallidus.

corresponds to type II neurons described by Nambu et. al. and to type A neurons described by Cooper et al. We have attempted to correlate cellular morphologies and relative location in the GP with the two neuronal populations but failed to find any consistent distinctions between them (Figure 2). In our preparation, type II GP neurons constitute about 80% of all neurons encountered and so most of our studies have focused on this neuronal population.

We report that the majority of GP neurons express both mGluR1a and mGluR5 (Figure 3). Both receptors are very abundant in the neuropil as well as perikarya in the rat GP. Stimulation with the group I-selective agonist (R,S)-3,5-DHPG (1-100 μ M) elicits a robust depolarization which, at maximum, reaches 15.7±2.1 mV in both neuronal populations in the GP (Figure 4). This depolarization is accompanied by a decrease in input resistance in both type I and type II neurons.

Data on post-synaptic expression of mGluRs in the GP predicts that only activation of group I mGluRs would elicit a measurable post-synaptic response in GP neurons (Kosinski et al., 1999), (Testa et al., 1998), (Bradley et al., 1999; Hanson and Smith, 1999). Indeed, group II and III selective agonists, LY354740 and LAP4, have no effect on the membrane potential or the input resistance of the rat GP neurons. We also report that DHPG-induced depolarization is solely mediated by mGluR1. MGluR1-selective antagonist, LY367385, completely abolishes the response to DHPG.

Interestingly, blockade of mGluR5 with the selective antagonist MPEP potentiates the response of type II GP neurons to DHPG. When activated alone, by an mGluR5 selective partial agonist CHPG, there is no change in the membrane potential of GP neurons. This suggests that mGluR5 may be involved in the desensitization of mGluR1. Such functional interaction between receptors of the same family is quite novel and has not been described for any other receptor groups. We are currently investigating the nature of this interaction.



Figure 3. Co-localization of mGluR1a and mGluR5 in the GP neurons. A. Low power confocal image of mGluR1a staining in GP. **B.** High power images of three GP neurons that co-localize mGluR1a and mGluR5. Fifty μ m thick horizontal brain slices were incubated with monoclonal α -mGluR and polyclonal α -mGluR5. Donkey IgGs conjugated to α -mouse Cy3 or rhodamine were used to detect mGluR1 staining. Donkey α -rabbit FITC-conjugated IgGs were used as secondary antibodies to detect mGluR5 immunoreactivity. Slices through GP were imaged on a fluorescent confocal microscope and analyzed using Adobe Photoshop. Str- striatum, GP – globus pallidus.



Figure 4. Effect of DHPG on the membrane potential of a type I GP neuron (A) and a type II GP neuron (B). All experiments were done in the presence of $0.5 \,\mu$ M TTX. Cells were held at -65 mV prior to application of DHPG. A line above the recording indicates time of the drug in the bath.

6. CONCLUSION

We are only beginning to understand the functions of mGluRs in the GP. So far, we have been able to show that activation of post-synaptic mGluR1 elicits a robust depolarization in GP neurons that is accompanied by a decrease in input resistance. At the same time, mGluR1a and mGluR5, which co-localize to the same neurons in the GP, are involved in a novel mode of interaction where mGluR5 desensitizes mGluR1-activated cascade.

The functions of mGluRs have been characterized in some nuclei of the basal ganglia. Most notably, mGluR5 directly depolarizes STN neurons where it also potentiates NMDA currents (Awad et al., 2000). Since the STN is overactive in Parkinson's disease (PD), mGluR5 antagonists have emerged as potential therapeutics in treating parkinsonian symptoms. The new function of mGluR5 in the GP reported in this study provides further evidence that mGluR5 antagonists, such as MPEP, would be beneficial in alleviating PD symptoms. In PD, GP is strongly inhibited by the Str. Blocking mGluR5 in the GP will increase the response of mGluR1 to glutamate thereby increasing the activity of underactive GP. Recent findings indicate that acute treatment with MPEP, indeed, produces antiparkinsonian-like effects in haloperidol-treated rats (Ossowska et al., 2001). Chronic treatment with MPEP has also been shown to ameliorate motor deficits in rat models of PD (Spooren et al., 2001).

The mechanisms by which mGluRs modulate the activity of GP neurons may be very complex. Since known down-stream effectors of mGluR activation can have profound and long-lasting consequences on the excitability of neurons, it is very likely that these receptors have vital functions in the GP. Since GP is an integrator of striatal and subthalamic inputs and it projects to many nuclei in the basal ganglia, modulating its

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activity should profoundly impact the processing of information flow through the basal ganglia circuit.

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APPENDIX V

Activation of Group II Metabotropic Glutamate Receptors Inhibits Synaptic Excitation of the Substantia Nigra Pars Reticulata

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Loss of nigrostriatal dopaminergic neurons in Parkinson's disease (PD) leads to increased activity of glutamatergic neurons in the subthalamic nucleus (STN). Recent studies reveal that the resultant increase in STN-induced excitation of basal ganglia output nuclei is responsible for the disabling motor impairment characteristic of PD. On the basis of this, it is possible that any manipulation that reduces activity at excitatory STN synapses onto basal ganglia output nuclei could be useful in the treatment of PD. We now report that group II metabotropic glutamate receptors (mGluRs) are presynaptically localized on STN terminals and that activation of these receptors inhibits excitatory transmission at STN synapses. In agreement with the

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. The primary pathological change giving rise to the symptoms of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta that modulate the function of neurons in the striatum and other nuclei in the basal ganglia (BG) motor circuit. Currently, the most effective pharmacological agents for the treatment of PD include levodopa (L-DOPA), the immediate precursor of dopamine, and other drugs that replace the lost dopaminergic modulation of BG function (Poewe and Granata, 1997). Unfortunately, dopamine replacement therapy ultimately fails in most patients because of loss of efficacy with progression of the disease and severe motor and psychiatric side effects (Poewe et al., 1986). Because of this, a great deal of effort has been focused on developing new approaches for the treatment of PD.

Recent studies reveal that loss of nigrostriatal dopamine neurons results in a series of neurophysiological changes that lead to overactivity of a critical nucleus in the BG motor circuit termed the subthalamic nucleus (STN). The STN contains glutamatergic projection neurons that provide the major excitatory input to the globus pallidus internal segment (GPi) and the substantia nigra

S.R.B. and M.J.M. contributed equally to this work.

Correspondence should be addressed to Dr. P. J. Conn, Department of Pharmacology, Rollins Research Building, Atlanta, GA 30322. E-mail: pconn@emory.edu. Copyright © 2000 Society for Neuroscience 0270-6474/00/203085-10\$15.00/0 hypothesis that this could provide a therapeutic benefit in PD, a selective agonist of group II mGluRs induces a dramatic reversal of catalepsy in a rat model of PD. These results raise the exciting possibility that selective agonists of group II mGluRs could provide an entirely new approach to the treatment of PD. These novel therapeutic agents would provide a noninvasive pharmacological treatment that does not involve the manipulation of dopaminergic systems, thus avoiding the problems associated with current therapies.

Key words: substantia nigra pars reticulata; subthalamic nucleus; group II metabotropic glutamate receptors; Parkinson's disease; catalepsy; presynaptic inhibition

pars reticulata (SNr), the major output nuclei of the basal ganglia. Increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neu-. rons in the output nuclei. This glutamate-mediated overexcitation of BG output ultimately leads to a "shutdown" of thalamocortical projections and produces the motor impairment characteristic of PD (Wichmann and DeLong, 1997). Discovery of the pivotal role of increased STN-mediated excitation of the BG output nuclei in PD has led to a major focus on surgical approaches for treatment. For instance, lesions or high-frequency stimulation of the STN provides a therapeutic benefit to PD patients (Limousin et al., 1995). In addition, pallidotomy, a surgical lesion of the GP, produces similar therapeutic effects by reversing the impact of increased activity of STN neurons (Laitinen et al., 1992; Baron et al., 1996). Development of these highly effective neurosurgical approaches provides a major advance in our understanding of the pathophysiology of Parkinson's disease. However, surgical approaches are not widely available to Parkinson's patients. Because of their invasive nature, high cost, and the considerable expertise required, such treatment is reserved for patients that are refractory to dopamimetic therapy.

An alternative to surgical approaches to reducing the increased excitation of basal ganglia output nuclei in PD patients would be to use pharmacological agents that counteract the effects of overactivation of the STN neurons by reducing transmission at excitatory STN synapses onto the SNr and GPi neurons. Although antagonists of postsynaptic ionotropic glutamate receptors can improve parkinsonian symptoms in PD patients and in animal models of PD (Klockgether et al., 1993; Kornhuber et al., 1994), these compounds are most effective as adjuncts to dopamine replacement therapy (Starr, 1995). Another approach would be to target metabotropic glutamate receptors (mGluRs), which are often localized presynaptically on glutamatergic terminals where

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they can inhibit glutamate release. Interestingly, the group II mGluRs (mGluR2 and mGluR3) are expressed in STN neurons (Testa et al., 1994), and these receptors have been shown to regulate glutamate release in other brain regions (Hayashi et al., 1993; Shigemoto et al., 1997). We now report that group II mGluRs are presynaptically localized on STN terminals in the SNr and that activation of these receptors reduces excitatory synaptic responses. Furthermore, activation of group II mGluRs provides a dramatic therapeutic effect in a rat model of Parkinson's disease. If this or related drugs prove to be effective in patients with Parkinson's disease, this could lead to a novel approach for the treatment of this debilitating disorder.

MATERIALS AND METHODS

Materials. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (R,S)- α -cyclopropyl-4-phosphonophenylglycine (CPPG), D(-)-2-amino-5-phosphonopentanoic acid (D-AP-5), and (2S,2'R,3'R)-2-(2',3'-dicarboxyc-clopropyl)glycine (DCG-IV) were obtained from Tocris (Ballwin, MO). 2R,4R-4-Aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC), (+)-2-aminobicyclo[3:1:0]-hexane-2,6-dicarboxylate monohydrate (LY354740), and 2S-2-amino-2-(1S,2S-2-carboxycclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495) were gifts from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). All other materials were obtained from Sigma (St. Louis, MO).

Electrophysiology. Whole-cell patch-clamp recordings were obtained as described previously (Marino et al., 1998) except that recordings were made under visual control. Fifteen- to 18-d-old Sprague Dawley rats were used for all patch-clamp studies. Brains were rapidly removed and submerged in an ice-cold sucrose buffer (in mM); sucrose, 187; KCl, 3; CaCl₂, 2; MgSO₄, 1.9; KH₂PO₄, 1.2; glucose, 20; and NaHCO₃, 26; equilibrated with 95% $O_2/5\%$ CO₂. Parasagittal slices (300 μ m thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber containing normal artificial CSF (ACSF; in mM, NaCl, 124; KCl, 2.5; MgSO₄, 1.3; NaH₂PO₄,1.0; CaCl₂, 2.0; glucose, 20; and NaHCO₃, 26; equilibrated with 95% O₂/5% CO₂). In some experiments, 5 μ M glutathione, 500 μ M pyruvate, and 250 μ M kynurenate were included in the sucrose buffer and holding chamber. These additional compounds tended to increase slice viability but did not have any effect on experimental outcome. Therefore all of the data from these two groups have been pooled. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room temperature ACSF (~3 ml/min; 23-24°C). Neurons in the substantia nigra pars reticulata were visualized with a 40× water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller (Tokyo, Japan) and filed with buffer (in mM, potassium gluconate, 140; HEPES, 10: NaCl, 10; EGTA, 0.6; GTP, 0.2; and ATP, 2; pH adjusted to 7.5 with 0.5N NaOH). Biocytin (0.5%; free base) was added just before use. Electrode resistance was 3-7 M[SCAP] Ω . For measurement of synaptically evoked currents, bipolar tungsten electrodes were used to apply stimuli to the STN. Stimulating electrodes were positioned with one pole slightly penetrating the tissue and the other pole above the slice. Synaptically evoked EPSCs were record from a holding potential of -60 mV, and slices were bathed in 50 µM picrotoxin. IPSCs were evoked in a similar manner but with the electrodes placed in the cerebral peduncle rostral to the recording sight and in the presence of 10 μM CNQX and 10 μM D-AP-5 to block excitatory transmission. IPSCs were recorded from a holding potential of -50 mV. STN-evoked fiber volleys were recorded by placing a low-resistance pipette $(0.5-2 M[scar]\Omega)$ filled with 3 M NaCl in the cerebral peduncle and stimulating the STN as described above. Fiber volleys were evoked in the presence of 20 μ M CNQX and 20 μ M bicuculline. For measurement of kainateevoked currents, kainate (100 μ M) was pressure ejected into the slice from a low-resistance pipette. Kainate-evoked currents were recorded from a holding potential of -60 mV, and slices were bathed in ACSF containing 500 nM tetrodotoxin. For studies of miniature EPSCs (mEP-SCs), slices were bathed in standard ACSF with the addition of 50 mM mannitol, 500 nm tetrodotoxin, and 10 µm bicuculline warmed to 25°C. Glutamate-evoked EPSCs were recorded in the presence of 20 µM bicuculline. Glutamate (100 µM in ACSF) was applied by a syringe pump (1 ml/min) through a microapplicator made from a fused silica microtube (MicroFil; WPI). The microapplicator was positioned slightly above the slice and dorsal to the STN. The flow of glutamate was parallel to the

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bath flow, and the slice was arranged so that glutamate application to surrounding areas was minimized (see Fig. 3). This method was also used to produce a local application of LY345740 for some experiments (see Fig. 1A,B). GABAergic projection neurons were identified according to previously established electrophysiological and morphological criteria (Richards et al., 1997). GABAergic neurons exhibited spontaneous repetitive firings, short-duration action potentials (half-amplitude duration = 1.7 ± 0.2 msec), little spike frequency adaptation, and a lack of inward rectification, whereas dopaminergic neurons displayed no, or low-frequency, spontaneous firings, longer-duration action potentials (half-amplitude duration = 7.0 ± 0.5 msec), strong spike frequency adaptation, and a pronounced inward rectification. Light microscopic examination of biocytin-filled neurons indicated that GABAergic neurons had extensive dendritic arborizations close to the cell body, whereas the dendritic structures of dopaminergic neurons were relatively sparse. All of the data presented in these studies are from confirmed GABAergic neurons.

Immunocytochemistry. Preparation of the tissue for immunocytochemical analysis at the electron microscopy level followed previously published protocols (Bradley et al., 1996). The avidin-biotin-peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used to detect mGluR2/3 immunoreactivity in rat (n = 2) SNr. The peroxidase reaction was developed in 0.05% diaminobenzidine and 0.01% H₂O₂. Antibodies that specifically recognize mGluR2 and mGluR3 are from Chemicon (Temecula, CA).

Behavioral studies. Male Sprague Dawley rats 30 d old at the start of experiments were injected intraperitoneally with either haloperidol (2 mg/ml solution dissolved in 8.5% lactic acid, neutralized with 1N NaOH, and diluted to 0.3 mg/ml in saline) or saline and returned to their home cage for 30 min. After 30 min, the animals were injected with either saline or LY354740 (0.6-6 mg/ml dissolved in saline). Catalepsy was measured 1 hr later by placing the animal's forepaws on a bar elevated 4.5 cm. The time to removal of one paw was measured by a stopwatch. Animals were then placed on a vertical mesh \sim 6 inches above the ground, and the time to remove one paw from the mesh was measured. Animals were tested once per day, and saline controls were run between each drug test. All animals were habituated to the tasks by 3 consecutive days of saline control treatment before beginning drug testing.

RESULTS

Whole-cell patch-clamp techniques were used to record EPSCs from GABAergic projection neurons of the SNr in midbrain slices. EPSCs were elicited by stimulation of the STN with bipolar stimulating electrodes (0.4–12.0 μ A every 60–90 sec) in the presence of 50 μ M picrotoxin. EPSCs elicited with this protocol had a constant latency, were monophasic, and were completely abolished with the application of 10 μ M CNQX (n = 10; data not shown), suggesting that the synaptic response was a monosynaptic glutamatergic EPSC.

Activation of group II mGluRs inhibits transmission at the STN–SNr synapse

Brief local application of 100 nm LY354740, a highly selective agonist of group II mGluRs (Monn et al., 1997; Schoepp et al., 1997), produced a reversible depression of EPSCs in SNr projection neurons (Fig. 1A,B). It should be noted, whereas LY354740 reduced evoked EPSCs in every cell tested, that longer bath applications resulted in inconsistent washout of the effect of LY354740. This is primarily because we recorded from cells at different depths in the slice. The deeper cells required longer periods of agonist application and exhibited slower washout of effects. However, longer bath applications were used in all additional studies to ensure an equilibrium concentration of drug at the sites of action and a maximal response. A concentrationresponse curve for LY354740 revealed an EC₅₀ of \sim 75 nM (Fig. 1C), consistent with the potency of this compound at group II mGluRs. The steep slope of the concentration-response curve for LY354740 is consistent with the dose-response relationship reported for a number other effects of this drug in both recom-
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Figure 1. Activation of group II mGluRs reduces EPSCs at the STN-SNr synapse. A, Evoked EPSCs before (Control), during (LY354740), and after (Wash Out) a brief local application of LY354740. Applications of LY354740 dramatically reduce EPSCs, and this effect is reversible. B, Average time course of the effect of 100 nm LY354740 (application indicated by horizontal bar). Each point represents the mean (± SEM) of data from five cells. C, Dose-response relationship of LY354740-induced inhibition of EPSCs. The effect of inhibition of EPSCs is maximal at 100 nm. Each point represents the mean of three experiments. D, Effects of specific group II mGluR agonists on EPSCs at the STN-SNr synapse and block of the LY354740-induced inhibition of EPSCs by application of group II mGluR antagonists before application of the agonist. Agonists include LY354740 (100 nM), APDC (100 μ M), and DCG-IV (3 μ M). Antagonists include LY341495 (100 nm) and CPPG (500 μ M). Each vertical bar represents the mean (± SEM) of data collected from five cells (*p < 0.01).

binant and native systems (Monn et al., 1997; Schaffhauser et al., 1997; Schoepp et al., 1997). The reduction of EPSC amplitude was mimicked by two other highly selective agonists of group II mGluRs, 2R, 4R-APDC (Schoepp et al., 1995) and DCG-IV (Hayashi et al., 1993; Gereau and Conn, 1995a) (Fig. 1*D*), and was completely blocked by previous application of LY341495 (100 nM) or CPPG (500 μ M) (Fig. 1*D*), both of which are antagonists active at group II mGluRs (Toms et al., 1996; Kingston et al., 1998).

Group II mGluRs are localized presynaptically at excitatory terminals in the SNr

Taken together, these data suggest that activation of group II mGluRs reduces transmission at the STN-SNr synapse. We used a combination of immunocytochemical and biophysical approaches to determine whether group II mGluRs elicit this effect by a presynaptic or a postsynaptic mechanism of action. First, we used antibodies that specifically recognize both mGluR2 and mGluR3 for immunocytochemical localization of group II mGluRs in the SNr. Analysis of mGluR2/3 immunoreactivity at the electron microscopic level revealed that group II mGluRs are presynaptically localized (Fig. 2). The morphology of the labeled

synapses, including their asymmetric nature, was characteristic of STN terminals (Fig. 2A-D) (Bevan et al., 1994). Quantification of the labeling was assessed by counting asymmetric terminals on three randomly selected grids that resulted in an estimated 30% labeling of asymmetric terminals (25 labeled terminal of 82 total). However, it is important to note that quantification of any preembedding immunocytochemical labeling at the electron microscopic level is confounded by the nonhomogeneous penetration of the antibodies through the vibratome sections. The first 5-10 μ m on both sides of the sections are usually labeled, whereas the middle remains devoid of immunostaining. This implies that the lack of immunoreactivity in some structures could be attributable either to a genuine lack of antigens or to the inaccessibility of the antibodies to this particular site. Therefore, only the positive immunolabeling can be conclusively interpreted. Because of this, the 30% labeling observed in these studies represents a lower limit to the extent of staining. We also observed labeling of terminals that did not make clear synaptic contact with postsynaptic elements and of fine processes that were reminiscent of previous reports of mGluR2/3 distribution in preterminal axons

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Figure 2. Group II mGluRs are presynaptically localized at asymmetric terminals in the SNr. A-D, Electron micrographs demonstrating presynaptic mGluR2/3 immunoreactivity at asymmetric terminals in the SNr. Labeled (*) axon terminals (t) are shown synapsing on unlabeled dendrites (d) and dendritic spines (s). E, An example of a labeled terminal forming a symmetric synapse. Synapses are indicated by arrows. Scale bar: A, 301 nm; B, 203 nm; C, 315 nm; D, 263 nm; E, 207 nm.

(data not shown) (Lujan et al., 1997). Furthermore, there was occasional labeling of symmetric synapses (Fig. 2E), although the majority of symmetric synapses were unlabeled. There was no observable staining of dendrites, dendritic spines, or other postsynaptic elements.

The group II mGluR-mediated inhibition of synaptic transmission is caused by a presynaptic mechanism

The presence of mGluR2/3 immunoreactivity at presynaptic but not postsynaptic sites in the SNr suggests that these receptors are likely to act by inhibiting glutamate release from presynaptic terminals rather than by modulating the postsynaptic glutamategated ion channels. To test this hypothesis further, we determined the effects of maximal concentrations of LY354740 on currents elicited by brief (50–500 msec) pressure ejection of kainate (100 μ M) into the slice. In the presence of 500 nM tetrodotoxin, kainate application produced a robust, stable, inward current in SNr GABAergic neurons (Fig. 3A). The kainateevoked currents were blocked by application of 10 μ M CNQX (n = 4; data not shown) indicating that they were mediated by activation of AMPA/kainate receptors. Application of 100 nM LY354740 produced no significant change in kainate-evoked currents (Fig. 3A,B).

Although the lack of effect of LY354740 on kainate-evoked currents is consistent with a presynaptic mechanism of action, it is conceivable that exogenously applied kainate selectively activates nonsynaptic glutamate receptor channels and that LY354740 selectively modulates channels that are localized at synapses. Thus, we also determined the effect of maximal concentrations of LY354740 on the frequency and amplitude of spontaneous mEPSCs. Recordings were made in the presence

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Figure 3. Activation of group II mGluRs has no effect on the response to exogenously applied kainate. A, Representative *traces* of kainate-evoked currents in the SNr projection neurons before (*Control; left*) and during application of 100 nm LY354740 (*right*). B, Time course of the effect of LY354740 on the amplitude of kainate-evoked currents. C, Mean data demonstrating the lack of effect of group II mGluR activation on kainate-evoked currents (mean \pm SEM; p > 0.05; n = 5).

of tetrodotoxin (500 nM) to block activity-dependent release and of bicuculline (10 μ M) to block GABA_A-mediated synaptic currents. LY354740 (100 nM) produced no significant alteration in mEPSC frequency, amplitude, or waveform (Fig. 4*A*--*C*). This can be observed by a lack of effect of LY354740 on either the amplitude histograms (Fig. 4*C*) or the cumulative probability plots (Fig. 4*D*). Furthermore, overlay of an average of all mEPSCs before and after LY354740 application shows identical current amplitudes and kinetics between the two conditions (Fig. 4*B*). The average mEPSC frequency is 4.71 ± 0.79 Hz before drug application and 4.66 ± 0.8 Hz during application of 100 nm LY354740 (p > 0.05; n = 5). The average amplitude of mEPSCs was 9.2 ± 1.3 pA before and 8.4 ± 0.8 pA after LY354740 addition (p >0.05; n = 5).

The lack of effect on mEPSC amplitude and frequency is consistent with the group II mGluR-mediated inhibition in synaptic transmission having a presynaptic site of action. There are a number of potential mechanisms by which a receptor could act presynaptically to reduce action potential-dependent release without decreasing the frequency of mEPSCs. For instance, mEPSCs are thought to be voltage independent and therefore should be insensitive to modulation of presynaptic voltagedependent ion channels. If a receptor reduces transmission by inhibiting a presynaptic voltage-dependent calcium channel or increasing conductance through a voltage-dependent potassium channel rather than having some downstream effect on the release machinery, this may reduce evoked responses without affecting mEPSCs. This effect has been demonstrated at a variety of synapses where agents known to act presynaptically, such as cadmium, abolish evoked EPSCs but have no effect on either the frequency or amplitude of mEPSCs (Parfitt and Madison, 1993; Doze et al., 1995; Gereau and Conn, 1995b; Scanziani et al., 1995).

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Figure 4. Inhibition of EPSCs at the STN-SNr synapse is mediated by a presynaptic mechanism. A, Examples of mEPSCs before (Pre-Drug; left) and during application of 100 nm LY354740 (right). B, Overlayed averages of all mEPSCs recorded before and during LY354740 application, demonstrating the lack of effect on the amplitude and kinetics of mEPSCs. C, Amplitude histograms of mEPSCs before (left) and during application of 100 nm LY354740 (right). D, Cumulative frequency plots illustrating the lack of effect of LY354740 on mEPSC amplitude (left) and inter-event interval (right) (Kolmogorov-Smirnov test; p = 0.99). The data shown are representative of five separate experiments.

Although the analysis of the effects of LY354740 on mEPSCs is consistent with a presynaptic site of action, one concern with studies of mEPSCs is that it is impossible to identify the source of afferent fibers. This issue is particularly important in cases in which there is no observable effect on mEPSC frequency because of the possibility that the majority of mEPSCs arise from a separate population of afferents than those stimulated to produce evoked release. Although the majority of glutamatergic input to the SNr arises from the STN, several other regions including the pedunculopontine nucleus (Charara et al., 1996) and the nucleus raphe (Corvaja et al., 1993) provide a sparse projection accounting for a small percentage of asymmetric terminals in the SNr that could release glutamate. To address this issue, we applied glutamate directly to the STN to produce a selective activation of STN cell bodies without exciting fibers of passage (Fig. 5A). Application of glutamate (100 µM) to the STN produced an increase in the frequency of spontaneous EPSCs recorded in SNr neurons (basal, 4.8 ± 1.6 Hz; glutamate, 12.4 ± 5.7 Hz; n = 5) without affecting spontaneous EPSC amplitude (basal, 9.6 ± 1.2 pA; glutamate, 9.4 \pm 1.4 pA; n = 5). In agreement with a selective activation of cell bodies, movement of the glutamate application pipette slightly out of the STN to the cerebral peduncle had no effect on spontaneous EPSC frequency (ratio of glutamate/basal, application to STN, 3.0 ± 1.3 ; application to cerebral peduncle, 0.92 ± 0.1 ; n = 3) (Fig. 5A). To test for group II mGluR-mediated inhibition of transmission at the STN-SNr synapse, we determined the effects of maximal concentrations of LY354740 on the

frequency and amplitude of glutamate-evoked EPSCs. In agreement with the electrical stimulation results, we found that activation of group II mGluRs significantly reduced the frequency of glutamate-evoked EPSCs without affecting the amplitude or kinetics of the response (Fig. 5B-F).

Taken together, these data strongly support the hypothesis that activation of group II mGluRs decreases transmission at the STN-SNr synapse by a presynaptic mechanism. However, it is possible that a group II mGluR agonist could reduce evoked EPSCs by a mechanism that does not directly involve regulation of synaptic transmission, such as inducing a decrease in the excitability of the STN neurons or decreasing axonal conductance. To examine the mechanism of this presynaptic modulation further, we assessed the effects of maximal concentrations of LY354740 on the excitability of STN neurons. Whole-cell current-clamp recordings from STN neurons during application of 100 nm LY354740 indicate that activation of group II mGluRs has no effect on membrane potential (control infusion $\Delta V_m =$ -0.93 ± 0.34 mV; n = 4; LY354740 infusion $\Delta V_m = -0.99 \pm$ 0.83 mV; n = 7) (Fig. 6A,D) or input resistance (control, 671 ± 123.6 $M[SCAP]\Omega; LY354740, 665 \pm 129.4 M[SCAP]\Omega; n = 3)$ (Fig. (6B,D). We also applied a series of small depolarizing current injections to obtain an approximate estimate of the action potential threshold. Application of 100 nm LY345740 did not effect the lowest potential at which action potentials were observed (control infusion, $-48.3 \pm 1.28 \text{ mV}$; LY354740, $-48.7 \pm 1.55 \text{ mV}$; n = 4)

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Figure 5. Activation of group II mGluRs reduces the frequency of EPSCs evoked by glutamate application to the STN. A, A demonstration of the experimental paradigm used. Direct application of glutamate (100 μ M; 1 ml/min; 30 sec) to the STN produces approximately a threefold increase in EPSC frequency without affecting EPSC amplitude. Moving the microapplicator to a position above the cerebral peduncle (cp) produced no change in the frequency of EPSCs, indicating that the glutamate effect is caused by selective activation of STN neurons and not by fibers of passage. B, Examples of glutamateevoked EPSCs both before (left) and during the application of 100 nM LY354740 (right). C, Overlayed traces of average glutamate-evoked EPSCs before and during 100 nM LY354740 application indicating no change in the amplitude or kinetics of the responses. D, Cumulative frequency plots illustrating a lack of effect of LY354740 on amplitude (*left*; Kolmogorov-Smirnov test; p > 0.05) and a significant increase in interevent interval (right; Kolmogorov-Smirnov test; p < 0.01), indicating that LY345740 selectively reduces the frequency of glutamate-evoked EP-SCs. E, Frequency-amplitude histograms demonstrating a decrease in the frequency but no change in the mean amplitude of glutamateevoked EPSCs. F, Mean (± SEM) data demonstrating that glutamate induces approximately a threefold increase in frequency over basal values without altering amplitude. This glutamateevoked increase is significantly reduced by LY345740. Each vertical bar represents the mean (\pm SEM) of data collected from five cells (*p <ò.05).

(Fig. 6C,D). Therefore, these data indicate that the group II mGluR-mediated inhibition of transmission at the STN-SNr synapse cannot be explained by a decrease in the somatic excitability of the presynaptic neurons. We also recorded presynaptic fiber volleys by placing an extracellular recording electrode in the cerebral peduncle, the point of entry of STN fibers into the SNr, and electrically stimulating the STN. In the presence of blockers of fast glutamatergic (20 µM CNQX) and GABAergic (20 µM bicuculline) transmission, we recorded a robust negative inflection in the field that was sensitive to tetrodotoxin (500 nM), indicating that this is a measure of the firing of presynaptic axons (Fig. 6E-G). Application of 100 nm LY354740 had no effect on the presynaptic fiber volley, indicating that activation of group II mGluRs does not alter STN axonal excitability. Taken together, these data indicate that the group II mGluR-mediated reduction in transmission at the STN-SNr synapse is caused by a modulation of the presynaptic terminal or the preterminal axon.



Activation of group II mGluRs has no effect on inhibitory synaptic transmission in the SNr

If group II mGluRs selectively regulate transmission at STN synapses without altering transmission at inhibitory synapses in the SNr, agonists of these receptors would have a net inhibitory effect on excitatory drive through this portion of the basal ganglia circuit. The immunocytochemical data presented above suggest that mGluR2/3 immunoreactivity is not present on the majority of inhibitory synapses in the SNr, suggesting that group II mGluRs are not likely to modulate IPSCs in this region. To test this hypothesis directly, we determined the effect of LY354740 on evoked IPSCs recorded in SNr projection neurons. Consistent with previous reports (Radnikow and Misgeld, 1998), stimulation of the cerebral peduncle produced a robust, bicuculline-sensitive IPSC (Fig. 7). Application of a concentration of the group II mGluR agonist LY354740 that is maximally effective in reducing EPSCs had no effect on IPSC amplitude. These results suggest



Figure 6. Activation of group II mGluRs does not effect the excitability of STN neurons. A, Representative current-clamp recording demonstrating that application of 100 пм LY345740 does not alter membrane potential. B, Overlayed traces of responses to the injection of hyperpolarizing current demonstrating that LY354740 has no effect on input resistance. C. Representative traces of experiments in which small depolarizing current injections were used to determine the lowest potential at which an STN neuron would produce an action potential. Application of 100 nm LY354740 has no effect on this potential. D, Mean (± SEM) of data demonstrating the lack of effect of group II mGluR activation on membrane potential, input resistance, or lowest spike potential. Data are from three to seven cells per condition. E, F, Representative traces (E) and time course (F)demonstrating that LY354740 does not alter presynaptic fiber volleys evoked by stimulation of the STN. G, Mean (± SEM) of data from four independent experiments demonstrating that activation of group II mGluRs has no effect on presynaptic fiber volleys. The fiber volley is blocked by the application of 500 nM TTX indicating that the volley is a measurement of presynaptic axonal action potential.

that agonists of group II mGluRs will selectively inhibit excitatory transmission through the indirect pathway to the SNr without impacting direct GABA-mediated inhibition of SNr neurons.

Activation of group II mGluRs exhibits antiparkinsonian effects

The preceding data clearly demonstrate that group II mGluRs mediate a presynaptic inhibition of transmission at the STN-SNr synapse. Because overactivity at this synapse is thought to contribute to the motor dysfunction associated with PD and other hypokinetic disorders, we tested the hypothesis that activation of group II mGluRs would increase mobility in a rat model of parkinsonism using haloperidol-induced catalepsy (Ossowska et al., 1990; Schmidt et al., 1997). Two standard behavioral measures were used to assess catalepsy in rats treated with the dopamine receptor antagonist haloperidol. First, the front paws of control and experimental rats were placed on a horizontal bar (4.5 cm high), and the latency to remove a paw from the bar was measured. Second, rats were placed on a vertical grid, and the latency to remove a paw from the grid was measured (Kronthaler and Schmidt, 1996). Consistent with previous reports (Ossowska et al., 1990; Schmidt et al., 1997), haloperidol (1.5 mg/kg) induced a robust catalepsy that could be observed as an increase in latency with both behavioral measures (Fig. 8). Interestingly, haloperidol-induced catalepsy was reversed in a dose-dependent manner by intraperitoneal injection of the group II mGluR agonist LY354740. Injection of LY354740 alone had no significant effect on these behavioral measures.

DISCUSSION

We have found that group II mGluRs are presynaptically localized on STN terminals in BG output nuclei where they reduce transmission at STN-SNr synapses. Furthermore, a selective agonist of group II mGluRs has behavioral effects in rats that are consistent with an antiparkinsonian action. These data suggest that activation of group II mGluRs restores the normal function of BG circuits by acting at a point downstream of the striatum where dopamine receptor blockade occurs.

The finding that LY354740 alone had no effect on measures of



Figure 7. Activation of group II mGluRs has no effect on inhibitory transmission in the SNr. A, Representative traces of evoked IPSCs before (*Pre-Drug; left*) and during the application of 100 nm LY354740 (right). B, Time course of the effect of LY354740 on IPSC amplitude. C, Mean data demonstrating the lack of effect of group II mGluR activation on IPSC amplitude. Data represent the mean (\pm SEM) of seven separate experiments (p > 0.05).

catalepsy is interesting because of previous studies demonstrating that nonselective mGluR agonists can induce catalepsy (Kronthaler and Schmidt, 1996). Because LY354740 is highly selective for group II mGluRs, it is likely that this mGluR-induced catalepsy is caused by activation of another mGluR subtype. Consistent with this, LY354740 produces no effect on motor activity when administered alone (Helton et al., 1998) but reduces haloperidolinduced muscle rigidity (Konieczny et al., 1998). Furthermore, agonists of group I mGluRs have physiological and behavioral effects that suggest that agonists of these receptors are likely to have catalepsy-inducing effects (Sacaan et al., 1991; Kaatz and Albin, 1995).

Other potential sites of action of group II mGluR agonists

Taken together with previous studies revealing a critical role of the STN in parkinsonian states (Guridi and Obeso, 1997; Wichmann and DeLong, 1998), the results of the present anatomical and physiological studies suggest that the behavioral effects of LY354740 are at least partially attributable to an mGluR2/3mediated reduction in glutamate release from STN terminals. However, it is possible that actions of group II mGluR at other sites could also contribute to this effect. Although the distribution of group II mGluRs in other basal ganglia structures is somewhat limited, previous studies reveal that these receptors are present in the striatum (Testa et al., 1998) where they are involved in regulating transmission at corticostriatal synapses (Lovinger and McCool, 1995; Pisani et al., 1997). If group II mGluRs are preferentially involved in inhibiting synaptic excitation of striatal projection neurons that give rise to the indirect pathway, this could contribute to the overall behavioral effects of group II mGluR agonists. Also, it is possible that group II mGluRs present in motor regions outside of the basal ganglia, such as the cortex



Figure 8. Activation of group II mGluRs reverses catalepsy in an animal model of Parkinson's disease. The degree of haloperidol-induced catalepsy was measured as either latency to the first paw movement when the animal was placed on a vertical grid (A) or latency to remove a paw from a bar (B). Haloperidol (1.5 mg/kg, i.p.) induces a pronounced catalepsy that was reversed in a dose-dependent manner by LY354740 (3-30 mg/kg, i.p.) (*p < 0.05). LY354740 alone had no effect on either measure of catalepsy. Data shown represent the mean (± SEM) of data collected from eight animals.

(Neki et al., 1996) and thalamus (Ohishi et al., 1993), could contribute to the motor effects of group II mGluR agonists.

It is interesting to note that, in addition to projecting to basal ganglia output nuclei, STN neurons also project to the dopaminergic neurons of the SNc (Kita and Kitai, 1987; Iribe et al., 1999). Furthermore, glutamate has been implicated as an excitotoxic agent in PD (Albin and Greenamyre, 1992; Rodriguez et al., 1998), suggesting that increased excitatory drive to the SNc may contribute to the progressive loss of SNc dopaminergic neurons in PD. On the basis of this, if group II mGluRs are also involved in inhibiting transmission at STN synapses in the SNc, it is possible that agonists of these receptors could reduce the component of SNc neuronal death that is mediated by STN-induced excitotoxicity. Interestingly, previous immunocytochemical studies reveal that mGluR2/3 immunoreactivity is present in the SNc (Testa et al., 1998). Furthermore, physiological studies reveal that agonists of group II mGluRs inhibit evoked EPSPs in this region (Wigmore and Lacey, 1998). Although the source of the excitatory afferents regulated by group II mGluRs in the SNc was not defined, it is possible that these EPSCs are mediated in part by activity at STN terminals. These data raise the exciting possibility that group II mGluR agonists have the potential not only for Bradley et al. . Group II mGluRs in Substantia Nigra

reducing the symptoms of established PD but also for slowing the progression of PD. Future studies will be needed to define clearly the role of increased STN activity in contributing to progression of the disorder and to define rigorously the mGluR subtypes involved in regulating transmission at STN-SNc synapses.

Summary

The data presented suggest that group II mGluRs are presynaptically localized on STN terminals in the SNr and that activation of these receptors selectively reduces transmission at excitatory STN synapses in this region. Taken together with the behavioral data presented, these studies raise the exciting possibility that agonists of group II mGluRs may provide a novel, nonsurgical approach to the treatment of PD that bypasses the problems inherent with dopamine-replacement therapy. Furthermore, because group II mGluR agonists act downstream from nigrostriatal dopaminergic neurons, these compounds could be useful for the treatment of drug-induced parkinsonism in patients treated with haloperidol and other dopamine receptor antagonists that are used as antipsychotic agents. Finally, it is important to note that pallidotomy and inactivation of the STN are being explored as having therapeutic potential in other movement disorders, including dystonia and tardive dyskinesias (Vitek et al., 1998), and that increased activity in the STN is implicated in some forms of epilepsy (Deransart et al., 1996, 1998, 1999; Vercueil et al., 1998). Thus, it is conceivable that inhibition of excitatory transmission at the STN-SNr synapse with group II mGluR agonists could have broader therapeutic potential than that of L-DOPA and other drugs used for dopamine replacement in PD patients.

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APPENDIX VI

Activation of Group I Metabotropic Glutamate Receptors Produces a Direct Excitation and Disinhibition of GABAergic Projection Neurons in the Substantia Nigra Pars Reticulata

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A pathological increase in excitatory glutamatergic input to substantia nigra pars reticulata (SNr) from the subthalamic nucleus (STN) is believed to play a key role in the pathophysiology of Parkinson's disease. We present an analysis of the physiological roles that group I metabotropic glutamate receptors (mGluRs) play in regulating SNr functions. Immunocytochemical analysis at the light and electron microscopic levels reveal that both mGuR1a and mGluR5 are localized postsynaptically in the SNr. Consistent with this, activation of group I mGluRs depolarizes SNr GABAergic neurons. Interestingly, although both group I mGluRs (mGluR1 and mGluR5) are expressed in these neurons, the effect is mediated solely by mGluR1. Light presynaptic staining for mGluR1a and mGluR5

The basal ganglia are a richly interconnected group of subcortical nuclei involved in the control of motor behavior. The primary input nucleus of the basal ganglia is the striatum, and the primary output nuclei are the substantia nigra pars reticulata (SNr) and the internal globus pallidus (entopeduncular nucleus in nonprimates). The striatum projects to these output nuclei both directly, providing an inhibitory GABAergic input, and indirectly through the external globus pallidus and the subthalamic nucleus (STN). The STN provides excitatory glutamatergic input to the SNr. A delicate balance between this inhibition and excitation is believed to be critical for motor control, and disruptions in this balance are believed to underlie a variety of movement disorders (Wichmann and DeLong, 1997, 1998)

Although much effort has been directed at elucidating the connectivity of the direct and indirect pathways, less is known about the modulatory influence various transmitters may have on these pathways. Increasing evidence suggests that G-proteincoupled metabotropic glutamate receptors (mGluRs) may play an important role in the regulation of basal ganglia functions. To date, eight mGluR subtypes (mGluR1-mGluR8) have been

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was also observed in some terminals forming symmetric synapses and in small unmyelinated axons. Consistent with this, activation of presyhaptic mGluR1a and mGluR5 decreases inhibitory transmission in the SNr. The combination of direct excitatory effects and disinhibition induced by activation of group I mGluRs could lead to a large excitation of SNr projection neurons. This suggests that group I mGluRs are likely to play an important role in the powerful excitatory control that the STN exerts on basal ganglia output neurons.

Key words: substantia nigra pars reticulata; group I metabotropic glutamate receptors; movement disorders; slow excitatory postsynaptic potential; disinhibition; basal ganglia output nucleus

cloned and are classified into three major groups based on sequence homology, coupling to second-messenger systems, and agonist selectivity (for review, see Conn and Pin, 1997). Group I mGluRs (mGluR1 and GluR5) couple to G_q and phosphoinositide hydrolysis, whereas groups II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, and mGluR8) couple to G_i/G_o and related effector systems such as inhibition of adenylate cyclase. These mGluRs are widely distributed throughout the CNS in which they play important roles in regulating cell excitability and synaptic transmission.

Previous studies have shown that mGluRs are expressed throughout the basal ganglia (Testa et al., 1994, 1998; Kerner et al.,1997; Kosinski et al., 1998, 1999; Bradley et al., 1999a,b) and play important roles in the regulation of synaptic transmission in the SNr. For example, activation of presynaptic group II and III mGluRs inhibits excitatory transmission at the STN-SNr synapse (Bradley et al., 2000; Wittmann et al., 2000). One of the major postsynaptic effects of mGluRs in many brain regions is a group I mGluR-mediated slow depolarization (Crepel et al., 1994; Guerineau et al., 1994, 1995; Gereau and Conn, 1995a; Miller et al., 1995). Because glutamatergic innervation of the SNr from the STN plays an important role in motor control, an understanding of the roles mGluRs play in modulating SNr GABAergic neurons could provide important insight into the mechanisms involved in the regulation of SNr firing in both physiological and pathological states. We now report that activation of group I mGluRs produces an excitation of the SNr by two distinct mechanisms. Activation of postsynaptic mGluR1 induces a pronounced excitation of SNr GABAergic neurons that is mimicked by stimulation of excitatory afferents. In addition, activation of both mGluR1 and mGluR5

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produce a decrease in inhibitory transmission in the SNr, resulting in increased excitability of this crucial basal ganglia output nucleus.

MATERIALS AND METHODS

 $[R-(R^*,S^*)]$ -6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5 gm]isoquinolin-5-yl)furo[3,4-e]-1,3-benzodioxol-8(6H)-one (Bicuculline), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), p(-)-2-amino-5-phosphonopentanoic acid (p-AP-5), (RS)-3,5-dihydroxyphenylglycine (DHPG), (RS)-3-amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulfonic acid (2-hydroxysaclofen), u(+)-2-amino-4-phosphonobutyric acid (L-AP-4), and (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (L-Y367385) were obtained from Tocris Cookson (Ballwin, MO). (S)-(+)-2-(3'-Carboxy-bicyclo [1.1.1]pentyl-)glycine (CBPG) was obtained from Alexis Corp. (San Diego, CA). (+)-2-Aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740) was a gift from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). Methylphenylethynylpyridine (MPEP) and 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPC-COEt) were gifts from R. Kuhn (Novartis, Basel, Switzerland). All other materials were obtained from Sigma (St. Louis, MO).

Antibody characterization. The specificity of antibodies used in immunocytochemical studies was tested by immunoblotting of homogenates from cell lines expressing mGluR1 or mGluR5 and a variety of brain regions. Baby hamster kidney and human embryonic kidney cell lines, respectively, expressing mGluR1 and mGluR5 were grown in highglucose DMEM with 10% FBS and 1% penicillin-streptavidin. They were washed with cold PBS, pH 7.4 and lysed in a homogenization buffer consisting of a protease inhibitor cocktail (Sigma) diluted 1:100 in 2 mM EDTA and 2 mM HEPES, pH 7.4. Cells were then homogenized by hand with five strokes of a Teflon pestle in a glass homogenization tube. Membranes were isolated by first centrifuging for 5 min at $1000 \times g$. The supernatant was then spun for 30 min at $35,000 \times g$. Finally, the membrane-rich pellets were then resuspended in 0.5 ml of lysis buffer. For brain homogenates, adult male Sprague Dawley rats were deeply anesthetized with an intraperitoneal injection of chloral hydrate (700 mg/kg), and brains were rapidly removed and dissected on ice. Specific brain regions were homogenized, and the membranes were isolated using the same homogenization and centrifugation protocol. Protein concentration was measured using a BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were diluted 5:1 in an 6× SDS loading buffer containing 0.6 M DL-dithiothreitol. Proteins were separated on a standard 7.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 30 min at 25°C in PBS with 3% nonfat dry milk. They were subsequently probed with primary antibody at 1:10,000 in blocking buffer at 4°C for 24 hr with either purified mouse monoclonal IgG1 raised against the entire C terminus of human mGluR1a (PharMingen, San Diego, CA) or purified rabbit polyclonal IgG1 raised against the mGluR5 C terminus (KSSPKYDTLI-IRDYTNSSSSL; Upstate Biotechnologies, Lake Placid, NY). Membranes were next washed in PBS twice for 10 min, incubated with an HRP-conjugated secondary antibody: goat anti-mouse for mGluR1 and goat anti-rabbit for mGluR5. Both secondary antibodies were diluted 1:10,000 in blocking buffer and incubated for 1 hr at 25°C. Blots were then washed once for 5 min in TBS with 3% nonfat dry milk, twice for 5 min in TBS with 0.1% Tween 20, once for 5 min in TBS, and developed using the ECL chemiluminescent kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunocytochemistry. Two male Sprague Dawley rats were deeply anesthetized with ketamine (100 mg/kg) and dormitor (10 mg/kg) and transcardially perfused with cold, oxygenated Ringer's solution followed by 500 ml of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB) (0.1 M, pH 7.4) and 300 ml of cold PB. Next, the brain was removed from the skull and stored in PBS (0.01 M, pH 7.4) before being sliced on a vibrating microtome into 60 μ M transverse sections. These sections were then treated with 1.0% sodium borohydride for 20 min and rinsed in PBS.

The sections were preincubated at room temperature in a solution containing 10% normal goat serum (NGS), 1.0% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS for 1 hr. They were then incubated overnight at room temperature in a solution containing primary antibodies raised against synthetic peptides corresponding to the C terminus of either mGluR1a [PharMingen and Chemicon (Temecula, CA)] or mGluR5 (Upstate Biotechnologies) diluted at 0.5–1.0 mg/ml in a solution containing 1.0% NGS, 1.0% BSA, and 0.3% Triton X-100 in PBS. Next,

the sections were rinsed in PBS and transferred for 90 min at room temperature to a secondary antibody solution containing biotinylated goat-anti-rabbit IgGs (Vector Laboratories, Burlingame, CA) diluted 1:200 in the primary antibody diluent solution. After rinsing, sections were put in a solution containing 1:100 avidin-biotin-peroxidase complex (ABC; Vector Laboratories) for 90 min. The tissue was then washed in PBS and 0.05 $\,$ M Tris buffer before being transferred to a solution containing 0.01 $\,$ M imidazole, 0.0005% hydrogen peroxide, and 0.025% 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma) in Tris for 7-10 min. Sections were then mounted on gelatin-coated slides, dried, and coverslipped with Permount.

For electron microscope studies, the sections were treated with cryoprotectant for 20 min and transferred to a -80° C freezer for an additional 20 min. They were then thawed and treated with successively decreasing concentrations of cryoprotectant and finally PBS. The immunocytochemical procedure was the same as used for the light microscope, except that Triton X-100 was not used, and the incubation in the primary antibody was performed at 4°C for 48 hr.

After DAB revelation, the sections were processed for the electron microscope. They were first washed in 0.1 M PB for 30 min and then post-fixed in 1.0% osmium tetroxide for 10 min. After rinsing in PB, the tissue was dehydrated by a series of increasing concentrations of ethanol (50, 70, 90, and 100%). Uranyl acetate (1.0%) was added to the 70% ethanol to enhance contrast in the tissue. Next, the sections were exposed to propylene oxide and embedded in epoxy resin (Durcupan; Fluka, Buchs, Switzerland) for 12 hr. They were then mounted on slides, coverslipped, and heated at 60°C for 48 hr.

Four blocks (two for mGluR1a and two for mGluR5) were cut from the SNr and mounted on resin carriers to allow for the collection of ultrathin sections using an ultramicrotome (Ultracut T2; Leica, Nussloch, Germany). The ultrathin sections were collected on single-slot copper grids, stained with lead citrate for 5 min to enhance contrast, and examined on a Zeiss (Thornwood, NY) EM-10C electron microscope.

Electron micrographs were taken at $10,000-31,500 \times$ magnification to characterize the nature of immunoreactive elements in the SNr.

Electrophysiology. Whole-cell patch-clamp recordings were obtained as described previously (Marino et al., 1998; Bradley et al., 2000). Fifteento 18-d-old Sprague Dawley rats were used for all patch-clamp studies. After decapitation, brains were rapidly removed and submerged in an ice-cold sucrose buffer [in mM: 187 sucrose, 3 KCl, 1.9 MgSO₄, 1.2 KH₂PO₄, 20 glucose, and 26 NaHCO₃ (equilibrated with 95% O₂-5% CO_2)]. Parasagittal or horizontal slices (300 μ m thick) were made using a Vibraslicer (World Precision Instruments, Sarasota, FL). Slices were transferred to a holding chamber containing normal artificial CSF (ACSF) [in mM: 124 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 2.0 CaCl₂, 20 glucose, 26 NaHCO₃ (equilibrated with 95% O₂-5% CO₂)]. In some experiments, 5 µM glutathione, 500 µM pyruvate, and 250 µM kynurenate were included in the sucrose buffer and holding chamber. These additional compounds tended to increase slice viability but did not have any effect on experimental outcome. Therefore, data from these two groups have been pooled. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continuously perfused with room temperature ACSF (~3 ml/min, 23-24°C). Neurons in the substantia nigra pars reticulata were visualized with a 40× water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige (Tokyo, Japan) vertical patch pipette puller and filled with the following (in mM): 140 potassium gluconate, 10 HEPES, 10 NaCl, 0.6 EGTA, 0.2 GTP, and 2 ATP (pH adjusted to 7.5 with 0.5N NaOH). Biocytin (0.5%, free base) was added just before use. Electrode resistance was 3-7 MΩ. For blockade of potassium channels, a modified ACSF was used (in mM: 108 NaCl, 19.6 NaC₂H₃O₂, 6 MgCl₂, 0.1 CaCl₂, 2.0 BaCl₂, 6.0 CsCl₂, 20 glucose, 26 NaHCO₃, 3 4-aminopyridine, and 25 tetraethylammonium chloride), and the intracellular solution was modified replacing the potassium gluconate with cesium methanesolfonate. For measurement of synaptically evoked slow EPSPs, ACSF was warmed to 34°C. Patch electrodes were filled with (in mM): 115 potassium methylsulfate, 5 HEPES, 20 NaCl, 1.5 MgCl₂, 0.1 EGTA, 2 Mg-ATP, 0.5 Na-GTP, and 10 phosphocretine (pH adjusted to 7.5 with 0.5 м KOH). Bipolar tungsten electrodes were used to apply stimuli to the SNr ~100 µm rostral to the recording site. Slow EPSPs were evoked in the presence of blockers of AMPA (10 µM CNQX), NMDA (10 µM D-AP-5), GABAA (50 μM picrotoxin), GABA_B (200 μM 2-hydroxysaclofen), dopamine (10 им haloperidol), and glycine (10 им strychnine) receptors. The stimulation parameters were 2-14 µA, 200 µsec, delivered in a train of 100-200 msec duration at a rate of 25-100 Hz. IPSCs were evoked with



Figure 1. The specificity of antibodies used for immunocytochemistry. Protein from cell lines expressing mGluR1a or mGluR5 or from homogenates of rat cerebellum (*Crb*), cortex (*Ctx*), hippocampus (*Hip*), and ventral midbrain (*V. Mid*) were separated by SDS-PAGE and transferred to membranes. The resulting blots were probed with either the monoclonal anti-mGluR1a (*A*) or anti-mGluR5 (*B*) antibodies as described in Materials and Methods. Each antibody specifically labels a band from the appropriate cell line and exhibits a distribution consistent with the known expression of the group I mGluRs. Similar results were observed with the polyclonal anti-mGluR1a antibody.

the stimulation electrode placed within the SNr rostrally or caudally to the recorded cell and recorded at a holding potential of -50 mV. CNQX (10-20 μ M) and 10-20 μ M D-AP-5 were continuously added to the bath to block excitatory transmission. To study miniature IPSCs (mIPSCs), the 140 mM potassium gluconate in the internal solution were substituted with 140 mM CsCl to reduce postsynaptic mGluR effects and increase currents. Therefore, inward mIPSCs were recorded at a holding potential of -80 mV in the presence 1 μ M tetrodotoxin (TTX).

Data analysis. All curve fitting was performed using the Marquardt-Levenburg algorithm as implemented in the SigmaPlot software package (SPSS, Chicago, IL). To determine an accurate reversal potential from the I-V ramps presented in Figure 6, the current-voltage relationships were fit with an arbitrary higher-order polynomial function of the form $I = I_0 + (C_1V) + (C_2V^2) \dots + (C_iV^i)$, where I is the whole-cell current, V is the command potential, I_0 is an offset variable, and C_i are constants. It was found that a third- order polynomial (i = 3) provided the best fit, with additional terms decreasing the error about the fit by <1%. Concentration-response curves were fit with a three-parameter Hill equation to obtain EC₅₀ and Hill slope values. All values are reported as mean \pm SEM.

RESULTS

Antibody specificity

To assess the specificity of the antibodies used in these studies, we performed immunoblot analysis on proteins isolated from cell lines and specific rat brain regions. As shown in Figure 1A, the anti mGluR1 monoclonal antibody (PharMingen) specifically labels a band at \sim 140 kDa in lanes containing protein from cells expressing mGluR1 but not from cells expressing mGluR5. In

addition, the mGluR1 antibody specifically labels a similar band in cerebellar homogenate, demonstrating a distribution consistent with previous reports (Martin et al., 1992; Shigemoto et al., 1992; Petralia et al., 1997). Similar results were observed with both mGluR1-selective antibodies used in these studies. In contrast to this, anti-mGluR5 polyclonal antibody specifically labels a similar band from cells expressing mGluR5 and, consistent with the known distribution of mGluR5, exhibits a broader labeling of brain homogenates in noncerebellar regions (Shigemoto et al., 1993; Romano et al., 1995) (Fig. 1*B*). In addition to these immunoblot studies, we also observed light level immunostaining for each antibody, consistent with previously reported distributions (data not shown).

Localization of group I mGluRs in the SNr

Previous studies have demonstrated the expression of both mGluR1 and mGluR5 in the SNr (Testa et al., 1994, 1998). However, these studies did not address the subcellular and subsynaptic localization of these receptors. To determine whether group I mGluRs are postsynaptically localized in the SNr, we performed immunocytochemical studies with antibodies selective for mGluR1a and mGluR5.

At the light microscopic level, the SNr exhibited labeling for both mGluR1a (Fig. 2A, B) and mGluR5 (Fig. 3A, B). To determine whether this immunoreactivity represents presynaptic or postsynaptic staining, we performed immunocytochemical analyses at the electron microscope level. Both antibodies primarily labeled dendritic processes that formed symmetric and asymmetric synapses with unlabeled terminals (Figs. 2,3). Although the majority of labeling was postsynaptic, immunoreactivity for both group I mGluRs was also found in small unmyelinated axons and a few axon terminals (Figs. 2C, 3C). In the case of presynaptic labeling for both group I mGluRs, the immunoreactivity was seen only in terminals forming symmetric synapses. A few glial processes were also labeled with both antibodies. Most immunoreactive dendrites were tightly surrounded by a large density of striatal-like terminals forming symmetric synapses (Figs. 2C,D; 3C-E), an ultrastructural feature typical of SNr GABAergic neurons (Smith and Bolam, 1991). In contrast, SNc dopaminergic neurons are much less innervated (Bolam and Smith, 1990). These data suggest that the majority of immunoreactive elements labeled with the two group I mGluR antibodies belong to SNr GABAergic neurons. Immunoreactive elements were counted in a random sample of SNr tissue to determine the relative frequency of group I mGluR-immunopositive elements. The relative distribution of mGluR1a immunoreactivity, expressed as a percentage of total labeled elements, was 64.2% dendrites, 34.3% axons, 0.4% somata, 0.8% terminals, and 0.4% glia. The relative distribution of mGluR5' immunoreactivity was 58.7% dendrites, 40.2% axons, 0.5% somata, and no observed labeling in terminals or glia.

Electrophysiological identification of GABAergic neurons in the SNr

For electrophysiological analysis of the roles of mGluRs in SNr GABAergic projection neurons, it is critical to differentiate between GABAergic neurons and the smaller population of dopaminergic neurons in this region. Fortunately, these two neuronal types exhibit distinct electrophysiological and morphological features. Therefore, we used electrophysiological criteria that were established previously to distinguish between dopaminergic neurons and GABAergic projection neurons (Nakanishi et al., 1987; 7004 J. Neurosci., September 15, 2001, 21(18):7001-7012

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Figure 2. mGluR1a immunoreactivity in the SNr. A, Low-power light micrograph of mGluR1a immunostaining in the SNc and SNr. B, Highpower light micrograph of mGluR1a-immunoreactive processes in the SNr. Lightly labeled neuronal cell bodies are indicated by *asterisks*. C, Low-power electron micrograph of mGluR1a-immunoreactive dendrites

Hausser et al., 1995; Richards et al., 1997). GABAergic neurons exhibit a high rate of spontaneous repetitive firing, short-duration action potentials (half-amplitude duration, 1.7 ± 0.2 msec; n = 4), little spike accommodation, and a lack of inward rectification (Fig. 4). In contrast, dopaminergic neurons display no or low-frequency spontaneous firing, longer-duration action potentials (halfamplitude duration, 7.0 ± 0.5 msec; n = 4), strong spike accommodation, and a pronounced inward rectification (Fig. 4). Light microscopic examination of biocytin-filled neurons indicated that GABAergic neurons had extensive dendritic arborizations close to the cell body, whereas dopaminergic neurons had sparser dendritic structure (data not shown). All data presented in this study are from electrophysiologically identified GABAergic neurons.

Activation of group I mGluRs depolarizes SNr GABAergic neurons

Previous studies have demonstrated that all three groups of mGluRs are expressed in the SNr (Testa et al., 1994, 1998). We therefore used maximal concentrations of group-selective mGluR agonists to determine whether activation of these receptors has an effect on membrane properties of SNr GABAergic neurons. In the presence of 0.5 µM TTX, application of the group I mGluRselective agonist DHPG induces a robust direct depolarization (300 μ M DHPG, 16.1 ± 2.6 mV; n = 5) of SNr neurons that reverses during drug washout (Fig. 5A, C). This depolarization is accompanied by a significant increase in input resistance (predrug, $498 \pm 70 \text{ M}\Omega$, n = 4; $100 \ \mu\text{M}$ DHPG, $619 \pm 89 \ \text{M}\Omega$, n = 4; p < 0.05; paired t test) (Fig. 5B), suggesting that a DHPGinduced decrease in membrane conductance underlies this effect. The concentration-response relationship for DHPG-induced depolarization of SNr GABAergic neurons exhibited a steep sigmoid shape and was fit with a Hill equation that gave an EC_{50} of 37 μ M and a Hill slope of 2.6 (Fig. 5D), consistent with an effect on group I mGluRs (Schoepp et al., 1994; Gereau and Conn, 1995a). In contrast to this group I mGluR-mediated depolarization, the group II-selective agonist LY354740 (Monn et al., 1997; Kingston et al., 1998) and the group III-selective agonist L-AP-4 (Conn and Pin, 1997) had no significant effect on resting membrane potential (Fig. 5A-C). Therefore, we focused on the physiology and pharmacology of the group I mGluR-mediated depolarization.

To determine the effect of group I mGluR activation on action potential firing in SNr GABAergic neurons, we applied the selective group I mGluR agonist DHPG in the absence of TTX. At the beginning of whole-cell recording, cells fire spontaneous action potentials (Fig. 4); however, within a few minutes, cells tend to hyperpolarize and do not fire spontaneously. Application of 100 μ M DHPG induced a robust depolarization and a large increase in action potential firing (Fig. 5*E*). This DHPG-induced firing is completely blocked by injection of hyperpolarizing current to maintain a -65 mV membrane potential during drug

⁽Den) in SNr. Note that the immunoreactivity is mostly found in dendritic processes but also occurs in small, unmyelinated axons (Ax) and a few axon terminals (Te). D, High-power electron micrograph of mGluRlaimmunoreactive dendrites that form asymmetric (arrowhead) and symmetric (arrow) synapses with unlabeled terminals. E, High-power electron micrograph showing an mGluRla-immunoreactive terminal in contact with a small, labeled dendrite. Note also the presence of an immunoreactive glial process (Gl) surrounding an unlabeled terminal. Scale bars: A, 500 μ m; B, 50 μ m; C, 1 μ m; D, E, 0.5 μ m.

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Figure 3. mGluR5-immunoreactive subtype within the SNr. A, Lowpower light micrograph of mGluR5 immunostaining in the SNc and SNr. B, High-power light micrograph of mGluR5-immunoreactive processes in the SNr. Labeled cell bodies are indicated by *asterisks*. C, Low-power electron micrographs of mGluR5-immunoreactive elements in the SNr.

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Figure 4. Demonstration of the identification of SNr GABAergic neurons. A, Response of a GABAergic (left) and dopaminergic (right) neuron to depolarizing and hyperpolarizing current injections. Note the pronounced spike frequency adaptation and inward rectification exhibited by the dopaminergic cell that is absent in the GABAergic cell. B, Examples of spike activity from resting cells. GABAergic neurons (left) fire at high frequency, whereas dopaminergic neurons (right) exhibit lower frequency or no spontaneous activity. C, Comparison of single action potentials from a GABAergic (left) and dopaminergic (right) neuron. All data presented here are from electrophysiologically identified GABAergic neurons.

application and is mimicked by direct depolarization of the cells to the same membrane potential (100 μ M DHPG, 3.8 \pm 0.3 Hz, n = 4; direct depolarization 3.2 \pm 0.7 Hz, n = 4; p > 0.05; Student's t test). These data suggest that the increase in firing is solely attributable to the depolarization and that mGluR activation does not have other effects on membrane properties of SNr neurons to increase firing frequency.

In other neurons, activation of group I mGluRs has been demonstrated to depolarize the cells by inhibition of a leak potassium conductance (Guerineau et al., 1994) or by an increase in a nonselective cationic conductance (Guerineau et al., 1995; Miller et al., 1995). Our observation that DHPG causes an increase in input resistance suggests that inhibition of leak potassium conductance is the most likely mechanism underlying this effect. Consistent with this, voltage-clamp analysis revealed a DHPG-induced inward current underlying the depolarization (Fig. 6A). Voltage ramps between -40 and -120 mV (20 mV/sec) were used to establish a current-voltage relationship of the

Note that the mGluR5 immunoreactivity is present in axonal (Ax) and dendritic process. D, E, High-power electron micrographs of mGluR5immunoreactive dendrites (Den) and spines (Sp) that form asymmetric synapses (arrowheads) with unlabeled terminals. Note the presence of an immunoreactive glial process (Gl). Scale bars: A, 500 μ m; B, 50 μ m; C, 1 μ m; D, E, 0.5 μ m.



Figure 5. DHPG induces a group I mGluR-mediated depolarization of SNr neurons. DHPG (100 μ M) induces a depolarization (A) and concomitant increase in input resistance in SNr GABAergic neurons (B). Maximal concentrations of the group II-selective agonist LY354740 and the group III-selective agonist L-AP-4 are without effect. C, Mean \pm SEM of data from five cells demonstrating that, at maximal concentrations, only the group I agonist DHPG induces a depolarization. D, Concentration-response relationship of the DHPG-induced depolarization. E, The effect of DHPG applied in the absence of TTX to demonstrate the robust increase in firing produced by activation of group I mGluRs.

DHPG-induced current. Application of 100 μ M DHPG induced a change in the slope of the whole-cell current-voltage relationship (Fig. 6B). Subtracting the trace in the presence of DHPG from the predrug *I*-*V* trace reveals the *I*-*V* relationship for the DHPG-induced current. This current was best fit with a third-order polynomial function (see Materials and Methods) (Fig. 6C). The interpolated reversal potential of $-111.7 \pm 7.4 \text{ mV}$ (n = 5) is in good agreement with the calculated Nernst equilibrium potential for potassium (-103.4 mV). In experiments in which cesium was included in both the intracellular and extracellular solutions and the ACSF included 4-aminopyridine and tetraethylammonium to block potassium channels, the DHPG-induced current was eliminated (Fig. 6C,D). Together, these data suggest that the DHPG-induced depolarization of SNr GABAergic neurons is mediated by decreasing a leak potassium conductance.

The DHPG-induced excitation of SNr GABAergic neurons is mediated by mGluR1

Our findings that both mGluR1a and mGluR5 are postsynaptically localized in SNr projection neurons suggests that both of these receptors could be involved in the DHPG-induced depoMarino et al. • Group I mGluRs in Substantia Nigra



Figure 6. Analysis of mGluR-mediated current in SNr GABAergic neurons. A, Application of 100 μ M DHPG induces an inward shift in holding current that reverses on drug washout. B, This inward shift is evident in the whole-cell current-voltage relationship determined by applying voltage ramps from -40 to -120 mV. C, Subtracting the trace in the presence of DHPG from the predrug *I-V* trace reveals an *I-V* relationship that reverses near the predicted potassium equilibrium potential. The solid line underlying the *trace* indicates the third-order polynomial fit described in Materials and Methods. Note that the inclusion of blockers of potassium channels inhibits this current. D, Mean \pm SEM of data from four cells in each condition comparing the DHPG-induced current recorded at a holding potential of -60 mV in control cells and in the presence of potassium channel blockers. *p < 0.01; t test.

larization. To determine the role each of these receptors plays in this effect, we used newly available pharmacological tools that distinguish between mGluR1 and mGluR5. CBPG, a partial agonist at mGluR5 that has antagonistic properties at mGluR1 (Mannaioni et al., 1999) failed to induce a depolarization at maximal concentrations (Fig. 7A,B), indicating that the depolarizing effect of DHPG is likely attributable to activation of mGluR1. Consistent with this, pretreatment with the highly selective, noncompetitive mGluR1 antagonist CPCCOEt (Annoura et al., 1996; Casabona et al., 1997; Litschig et al., 1999) or the highly selective, competitive mGluR1 antagonist LY367385 (Clark et al., 1997) produced a significant reduction in the DHPG-induced depolarization of SNr GABAergic neurons (Fig. 7A,B). Pretreatment with MPEP, a highly selective noncompetitive antagonist of mGluR5, had no significant effect at concentrations shown to be effective at blocking mGluR5 in other systems (Bowes et al., 1999; Gasparini et al., 1999) (Fig. 7A,B).

mGluR1 mediates a slow EPSP in SNr GABAergic neurons

The data presented thus far indicate that mGluR1 mediates direct excitation of SNr projection neurons. The SNr receives a sparse yet important glutamatergic innervation from the STN, and burst firing of the STN is known to play a key role in several neurological disorders, including Parkinson's disease (PD) (Hollerman and Grace, 1992; Bergman et al., 1994; Hassani et al., 1996). If



Figure 7. The group I mGluR-induced depolarization is mediated by mGluR1. A, Representative traces demonstrating that the DHPGinduced depolarization of SNr GABAergic neurons is not mimicked by the mGluR5-selective agonist CBPG. Furthermore, preincubation with the highly selective noncompetitive mGluR1 antagonist CPCCOEt or the highly selective competitive mGluR1 antagonist LY367385 fully blocks the DHPG-induced depolarization, whereas the mGluR5-selective antagonist MPEP is without effect. B, Mean \pm SEM of data from five cells per condition demonstrating the selective antagonism of the group I mGluR1-selective antagonist. *p < 0.01; Student's t test.

activation of glutamatergic afferents to the SNr release sufficient glutamate to activate mGluR1, the resulting excitation of SNr projection neurons could play an important role in these disease states. We tested this hypothesis by recording from SNr GABAergic neurons in the presence of ionotropic glutamate receptor and GABA receptor antagonists, as well as haloperidol to block dopamine receptors and strychnine to block glycine receptors. High-frequency stimulation of the afferents within the SNr produced a robust and reliable slow EPSP that reached threshold for action potential firing (Fig. 8A). Recent reports have demonstrated that, under carefully controlled conditions, synaptically released glutamate acting on group I mGluRs can induce a hyperpolarizing response in midbrain dopamine neurons (Fiorillo and Williams, 1998). Under the conditions used in these studies, we were able to elicit a hyperpolarizing response in four of four dopaminergic neurons recorded from the SNcventral tegmental area (Fig. 8A). However, we never observed a hyperpolarizing response in SNr neurons (0 of 22 cells). This suggests that depolarization is the primary action of glutamate acting on group I mGluRs on SNr GABAergic neurons. Consistent with mediation by mGluR1, this slow EPSP was reversibly blocked by 300 μM LY367385 (predrug, 9.0 ± 1.2 mV; LY367385, $3.9 \pm 0.7 \text{ mV}; n = 6; p < 0.05; \text{ paired } t \text{ test}$ (Fig. 8B,C), whereas J. Neurosci., September 15, 2001, 21(18):7001-7012 7007



Figure 8. mGluR1 mediates a slow EPSP in SNr GABAergic neurons. A, High-frequency stimulation of afferents in the SNr elicits a slow EPSP that exceeds action potential threshold and induces firing. Similar experiments in dopaminergic neurons of the SNc reveal a hyperpolarizing response; however, the only response observed in SNr GABAergic neurons is a depolarization. Representative traces (B) and mean \pm SEM data (C) demonstrating the inhibition of the slow EPSP by the mGluR1-selective antagonist LY367385. MPEP alone or in the presence of LY367385 is without effect. *p < 0.05; t test. This slow EPSP is fully blocked by 1 μ M TTX, suggesting that the residual slow EPSP in the presence of LY367385 is mediated by the release of some transmitter acting on a receptor other than a group I mGluR. Calibration in A has the same value as in B. Membrane potential in A was -50 mV. For experiments in B and C, membrane potential was manually held at -70 mV by current injection to avoid spiking and allow for accurate quantification.

the mGluR5-selective antagonist MPEP was without significant effect (predrug, 7.8 ± 1.0 mV; MPEP, 6.4 ± 0.9 mV; n = 6; p > 0.05; paired t test). Because it is possible that a small component of the slow EPSP is mediated by mGluR5, which is not detectable in the presence of the larger mGluR1-mediated component, we applied a combination of the two selective antagonist. This combination did not produce any inhibition greater than that observed with LY367385 alone (inhibition by LY367385, 44.8 \pm 5.6%, n = 6; inhibition by LY367385 plus MPEP, $46.5 \pm 4.2\%$, n = 4; p > 0.05; t test) (Fig. 8C). Interestingly, application of 1 μ M tetrodotoxin fully blocked the slow EPSP (predrug, 8.0 ± 1.0 mV; TTX, 0.1 ± 0.3 mV; n = 3; p < 0.05; paired t test), suggesting that the LY367385-insensitive component of the slow EPSP is mediated by the action potential-dependent release of neurotransmitter acting on a receptor other than the group I mGluRs.

Group I mGluRs decrease inhibitory transmission in the SNr

It was surprising that our immunocytochemical studies revealed presynaptic labeling in the SNr. In some other brain regions, mGluRs can act as heteroreceptors to reduce GABA release and



Figure 9. Activation of group I mGluRs decrease inhibitory transmission in the SNr. A, Representative traces of evoked IPSCs before (predrug), during (DHPG), and after washout of a brief bath application of 100 μ M DHPG. B, Average time course of the effect of 100 μ M DHPG; each point represents the mean \pm SEM of data from five cells. C, Dose-response relationship of DHPG-induced suppression of IPSCs. Each point represents the mean \pm SEM of three to four experiments.

inhibitory synaptic transmission. If activation of group I mGluRs decreases inhibitory transmission in the SNr, this combined with the direct excitatory effects described above would provide a mechanism whereby group I mGluR activation could exert a powerful excitatory influence on the SNr. We directly tested this hypothesis by recording IPSCs in SNr GABAergic projection neurons. IPSCs were evoked by stimulating within the SNr with bipolar stimulation electrodes (0.4-12.0 µA every 30 sec) and were recorded at a holding potential of -50 mV in the presence of AMPA (CNQX; 10-20 μм) and NMDA (D-AP-5; 10-20 μм) receptor antagonists to prevent excitatory synaptic transmission. Bicuculline (10 μ M; n = 8) abolished evoked IPSCs in all cells tested, confirming that the evoked currents were GABAA receptor-mediated responses. Short (3 min) bath application of the group I mGluR-selective agonist DHPG (100 μ M) reduced the amplitude of evoked IPSCs in a reversible manner (Fig. 9A.B). Concentration-response analysis revealed that the inhibition of IPSCs by DHPG was concentration dependent. The relationship was fit with a Hill equation that gave an EC_{50} value of 30 µM and Hill slope of 1.1. (Fig. 9C). This is consistent with the potency of DHPG on group I mGluRs.

Pharmacological studies of the DHPG-induced decrease in inhibitory transmission using subtype-selective antagonists were performed to determine which group I mGluR subtypes mediate this effect. The mGluR5-selective antagonist MPEP (10 μ M) had a slight tendency to block the DHPG-induced effect, but the response to MPEP did not reach statistical significance (n = 8; p > 0.05; t test) (Fig. 10*B*,*E*). In contrast, the mGluR-selective antagonist CPCCOEt induced a significant reduction of the DHPG-induced suppression of IPSCs (n = 8; p < 0.05; t test) (Fig. 10*C*,*E*). However, the response to CPCCOEt was only a partial blockade of the response, and DHPG still induced a



Figure 10. The group I mGluR-mediated decrease in inhibitory transmission involves both mGluR1 and mGluR5. A-D, Traces of evoked IPSCs before (control), during, and after (*Washout*) bath application of DHPG alone (A) or in the presence of selective antagonists (B-D). Selective antagonists include 10 μ M MPEP (mGluR5 selective; B) and 100 μ M CPCCOEt (mGluR1 selective; C) and the combination of both (D). E, Bar graph showing the average effect of selective antagonists on the DHPG-induced inhibition of IPSCs. Each bar represents the mean \pm SEM of data collected from eight cells. *p < 0.05; **p < 0.01.

 $20.9 \pm 4.6\%$ inhibition of IPSCs in the presence of this antagonist. Because neither antagonist was capable of completely blocking the response when added alone, we also determined the effect of a combination of both CPCCOEt and MPEP. The combination of antagonists completely blocked the ability of DHPG to reduce evoked IPSCs (n = 8; p < 0.01) (Fig. 10*D*,*E*), suggesting that both mGluR1 and mGluR5 may participate in regulation of IPSCs in SNr.

The group I mGluR-mediated decrease in inhibitory transmission occurs by a presynaptic mechanism

To determine whether the group I mGluR-mediated decrease in inhibitory transmission in the SNr is mediated through a presynaptic mechanism, we determined the effect of maximal concentrations of DHPG on frequency and amplitude of spontaneous mIPSCs. All mIPSC recordings were preformed at a holding potential of -80 mV in the presence of CNQX ($10-20 \ \mu M$) and p-AP-5 ($10-20 \ \mu M$) to block glutamatergic synaptic currents and 1 μM TTX to block activity-dependent release of transmitter. mIPSCs were measured as inward currents with pipettes in which Cl⁻ (140 mM) was the major anion in the internal solution.

Application of the group I selective agonist DHPG (100 μ M) had no significant effect on mIPSC frequency or amplitude (Fig.



Figure 11. Inhibition of IPSCs induced by the activation of group I mGluRs is mediated by a presynaptic mechanism. A, Examples of mIPSC traces before (predrug) and during application of 100 μ M DHPG. B, Amplitude histograms of mIPSCs before (*left*) and during (*right*) application of 100 μ M DHPG. C, Cumulative probability plots showing a lack of effect of DHPG on mIPSC amplitude (*left*) (Kolmogorov-Smirnov; p > 0.05) and interevent interval (*right*) (Kolmogorov-Smirnov; p > 0.05). Data shown are pooled from four experiments. D, Traces of paired-pulse experiments before (*Pre-drug*) and during application of 30 μ M DHPG. On the *right*, an overlay of the predrug trace (*straight line*) and a trace during application of DHPG scaled to the amplitude of the first IPSC (*dashed line*) is shown. DHPG increases the ratio of paired-pulse facilitation in five of six cells.

11A,B). This can be seen as a failure to induce a significant shift in the amplitude or interevent interval cumulative probability plots (amplitude, Kolmogorov-Smirnov, p > 0.05, n = 4; frequency, Kolmogorov–Smirnov, p > 0.05, n = 4) (Fig. 11B). The average mIPSC frequency before drug application was 1.74 ± 0.4 and 1.40 \pm 0.4 Hz after application of 100 μ M DHPG (p > 0.05; n = 4). The average mIPSC amplitude was 29.3 \pm 4.2 pA before and 31.9 \pm 3.5 pA after DHPG application (p > 0.05; n = 4). This lack of an effect on mIPSC amplitude and frequency is consistent with a presynaptic site of action for the group I mGluR-mediated suppression of synaptic transmission (Parfitt and Madison, 1993; Doze et al., 1995; Gereau and Conn, 1995b; Scanziani et al., 1995; Bradley et al., 2000). To further test this hypothesis, we also determined the effect of DHPG on pairedpulse facilitation of evoked IPSCs. All paired-pulse recordings were made in the presence of CNQX (10-20 µM) and D-AP-5 (10-20 μ M) with standard internal solution to allow measurement of outward IPSCs. IPSCs were evoked every 30 sec by paired stimulations of equal strength with a 50 msec interpulse interval. At these intervals, paired-pulse facilitation was observed in all recordings (60.2 \pm 6.3%; n = 11). Only cells that showed an agonist-induced inhibition of the amplitude of the first IPSC of at least 25% were used for analysis. DHPG (30 μ M) induced an increase in paired-pulse facilitation (Fig. 11D) in five of six cells examined. In those cells, the average increase in paired-pulse facilitation induced by DHPG was 56.1 \pm 11.7% (p < 0.05; n = 5) over the facilitation seen in the absence of DHPG.

Together, these studies suggest that activation of the group I mGluRs mGluR1 and mGluR5 reduce inhibitory transmission in the SNr through a presynaptic mechanism. Furthermore, this decrease in GABAergic inhibition may combine with the direct postsynaptic excitatory effects of mGluR1 activation to produce a powerful excitation of this crucial basal ganglia output nucleus.

DISCUSSION

The data presented here demonstrate that activation of group I mGluRs produces an excitation of the SNr. Both mGluR1 and mGluR5 are found at postsynaptic sites in the SNr and are sparsely localized in unmyelinated axons and putative GABAergic axon terminals in this region. Activation of group I mGluRs produces an excitation of SNr neurons by two distinct mechanisms. Activation of postsynaptically localized group I mGluRs on SNr GABAergic neurons produces a robust depolarization that induces a marked increase in action potential firing. The depolarization is accompanied by a decrease in membrane conductance, and the underlying current has a reversal potential consistent with mediation by inhibition of a leak potassium channel. Furthermore, this effect is attributable to selective activation of mGluR1 and can be produced by synaptically released glutamate. Activation of group I mGluRs also induces a decrease in inhibitory transmission in the SNr. This effect is mediated by both mGluR1 and mGluR5 and occurs through a presynaptic mechanism.

Because the glutamatergic projection from the STN provides a large proportion of excitatory terminals on SNr GABAergic neurons, it is likely that the primary source of glutamate acting on group I mGluRs is released from STN afferents. However, several other regions, including the pedunculopontine nucleus (Charara et al., 1996) and the nucleus raphe (Corvaja et al., 1993), provide a sparse projection accounting for a small percentage of asymmetric terminals in the SNr. Therefore, group I mGluRs may also modulate these inputs. Interestingly, although both mGluR1 and mGluR5 are postsynaptically localized in SNr neurons, our pharmacological studies demonstrate that activation of mGluR1 is solely responsible for the group I-mediated depolarization. This is of interest because both mGluR1 and mGluR5 couple to phosphoinositide hydrolysis and are capable of inducing depolarization of other neuronal populations (for review, see Conn and Pin, 1997; Anwyl, 1999). A potential explanation of this may be provided by recent immunogold studies examining the subcellular localization of the group I mGluRs in SNr (Hubert et al., 2001). This study observed that mGluR1a immunoreactivity is predominately associated with the membrane. In contrast, >80% of mGluR5 immunoreactivity was localized to a cytoplasmic compartment. Thus, specificity of function may be produced by differences in subsynaptic localization or some other functional segregation of these receptors. It should be noted that, whereas mGluR1 plays the predominate role in mediating the group I mGluR-induced depolarization in the SNr, mGluR5 may play

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important physiological roles regulating cell properties that were not measured in the present study. For example, group I mGluRs are known to modulate NMDA receptor currents in a variety of brain regions, and it is possible that mGluR5 is involved in a similar modulation in SNr. Future studies on the role of mGluR5 in these cells may provide important insight into the distinct functional roles of closely related receptor subtypes within a single neuronal population.

In addition to the postsynaptic labeling of neurons in the SNr for both group I mGluR subtypes, we also detected presynaptic staining. Consistent with this, we found that activation of presynaptic group I mGluRs decreases inhibitory transmission. The results of both the paired-pulse experiments and the analysis of mIPSCs strongly suggest that the group I mGluR-mediated decrease in IPSCs has a presynaptic mechanism of action, yet the relatively sparse staining detected in inhibitory terminals appears unlikely to be sufficient to mediate this response. The more abundant axonal staining may represent group I mGluRs on preterminal axons of GABAergic neurons, which could mediate the observed decrease in inhibitory transmission. It should be noted that this distribution is reminiscent of previous reports of mGluR2/3 distribution in preterminal axons at sites distant from the synapse (Lujan et al., 1997). On the other hand, the finding that the decrease in inhibitory transmission has a presynaptic locus does not necessarily require that the receptor mediating this response is localized presynaptically. For example, in the CA1 region of the hippocampus, depolarization of CA1 pyramidal neurons induces the release of a putative retrograde transmitter that decreases inhibitory transmission through a presynaptic mechanism (Alger et al., 1996). Our current experiments do not allow us to distinguish between such a mechanism and an action of DHPG on a presynaptically localized receptor.

The finding that group I mGluRs both directly excite and disinhibit SNr neurons is of particular interest for understanding the role the STN plays in modulation of the SNr. The indirect pathway is composed of striatal projections through the globus pallidus and the STN, which constitute a large percentage of excitatory terminals on SNr GABAergic neurons (Smith et al., 1998). Although the glutamatergic input to the SNr is sparse, it plays a critical role in basal ganglia functions, as evidenced by the pronounced clinical effects of STN lesions in PD (Guridi and Obeso, 1997). The STN also plays a key role in the pathological activity of the SNr. Transition of STN neurons from single-spike activity to burst-firing mode and resultant over excitation of the SNr has been implicated in the pathophysiology of PD (Hollerman and Grace, 1992; Bergman et al., 1994; Hassani et al., 1996), as well as some forms of epilepsy (Deransart et al., 1998). Furthermore, STN neurons exhibit extremely high firing rates and can typically exceed 25-50 Hz during burst-firing mode (Hollerman and Grace, 1992; Bergman et al., 1994; Wichmann et al., 1994; Beurrier et al., 1999; Bevan and Wilson, 1999). The robust excitatory effects of mGluR1 activation described here could play an important role in the powerful control exerted by the relatively sparse glutamatergic input to this nucleus from the STN.

Our current findings add to a growing body of literature suggesting that group I mGluRs play important roles in regulating functions of basal ganglia circuits (for review, see Smith et al., 2000, 2001; Conn et al., 2001; Rouse et al., 2001). For instance, mGluR5 is heavily expressed in the striatum and is also present at lower levels in the STN and the pallidal complex (Testa et al., 1994, 1995; Kerner et al., 1997; Tallaksen-Greene et al., 1998; Hanson and Smith, 1999). Although the levels of mGluR1 mRNA

are more limited, this receptor is also found throughout the basal ganglia (Testa et al., 1994; Kerner et al., 1997; Tallaksen-Greene et al., 1998; Hanson and Smith, 1999). A number of studies suggest that agonists of group I mGluRs may act at several levels to increase the net activity of projection neurons in basal ganglia. For instance, activation of group I mGluRs potentiates NMDA receptor currents in striatal neurons (Colwell and Levine, 1994; Pisani et al., 1997). Furthermore, behavioral studies combined with studies of changes in 2-deoxyglucose uptake and Fos immunoreactivity suggest that injection of group I mGluR agonists in the striatum induces a selective activation of the indirect pathway from the striatum and thereby increases activity of the output nuclei (Kaatz and Albin, 1995; Kearney et al., 1997). In addition, recent physiological studies suggest that activation of group I mGluRs has profound excitatory effects on STN projection neurons (Abbott et al., 1997; Awad and Conn, 1999). These previous studies together with the present data suggest that group I mGluRs function at three major sites to increase overall output of the basal ganglia motor circuit.

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Activation of Group III mGluRs Inhibits GABAergic and Glutamatergic Transmission in the Substantia Nigra Pars Reticulata

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Wittmann, Marion, Michael J. Marino, Stefania Risso Bradley, and P. Jeffrey Conn. Activation of group III mGluRs inhibits GABAergic and glutamatergic transmission in the substantia nigra pars reticulata. J Neurophysiol 85: 1960-1968, 2001. The GABAergic projection neurons of the substantia nigra pars reticulata (SNr) exert an important influence on the initiation and control of movement. The SNr is a primary output nucleus of the basal ganglia (BG) and is controlled by excitatory inputs from the subthalamic nucleus (STN) and inhibitory inputs from the striatum and globus pallidus. Changes in the output of the SNr are believed to be critically involved in the development of a variety of movement disorders. Anatomical studies reveal that metabotropic glutamate receptors (mGluRs) are highly expressed throughout the BG. Interestingly, mRNA for group III mGluRs are highly expressed in STN, striatum, and globus pallidus, and immunocytochemical studies have shown that the group III mGluR proteins are present in the SNr. Thus it is possible that group III mGluRs play a role in the modulation of synaptic transmission in this nucleus. We performed whole cell patch-clamp recordings from nondopaminergic SNr neurons to investigate the effect of group III mGluR activation on excitatory and inhibitory transmission in the SNr. We report that activation of group III mGluRs by the selective agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4, 100 µM) decreases inhibitory synaptic transmission in the SNr. Miniature inhibitory postsynaptic currents studies and paired-pulse studies reveal that this effect is mediated by a presynaptic mechanism. Furthermore we found that L-AP4 (500 μ M) also reduces excitatory synaptic transmission at the STN-SNr synapse by action on presynaptically localized group III mGluRs. The finding that mGluRs modulate the major inputs to SNr neurons suggests that these receptors may play an important role in motor function and could provide new targets for the development of pharmacological treatments of movement disorders.

INTRODUCTION

The basal ganglia (BG) is a highly interconnected group of subcortical nuclei in the vertebrate brain that plays a critical role in control of movement. The substantia nigra pars reticulata (SNr) is an important component of the basal ganglia motor circuit. The GABA containing projection neurons of the SNr together with those of the entopeduncular nucleus comprise the principal output nuclei of the BG (Grofova et al. 1982) that exert an important influence on the initiation of movement (Kilpatrick et al. 1982) and on motor control (Alexander and Crutcher 1990). Because of this, changes in the GABAergic output of the BG are believed to play an important role in physiological as well as in pathophysiological conditions.

Inhibitory output from the SNr is controlled by two opposing but parallel pathways (Bergman et al. 1990; DeLong 1990). The "direct pathway" originates from a subpopulation of GABAergic striatal neurons that project directly to the SNr and thereby inhibit activity in these output neurons. The "indirect pathway" originates from a different population of GABAergic striatal neurons that project to the SNr via the external segment of the globus pallidus and the subthalamic nucleus (STN), providing an excitatory glutamatergic input to SNr neurons. An intricate balance of activity between these pathways is believed to be necessary for a normal fine tuning of motor function, and the disruption of this balance leads to various movement disorders (Wichmann and DeLong 1997, 1998). Hypokinetic movement disorders such as Parkinson's disease are produced by a relative increase in BG output mediated by a decrease in activity of inhibitory inputs via the direct pathway and an increase in activity of excitatory inputs through the indirect pathway. A relative decrease of BG output, on the other hand, leads to the development of hyperkinetic disorders including Huntington's disease and Tourette syndrome. Furthermore inhibition of GABAergic SNr projection neurons has been shown to result in suppression of seizures in various animal models of epilepsy (Deransart et al. 1998). Since the output of the SNr is so critically involved in normal as well as pathological brain processes, receptors that modulate excitatory and inhibitory inputs to SNr neurons could provide important targets for drug development. One family of receptors that may provide such a target are the metabotropic glutamate receptors (mGluRs).

Metabotropic glutamate receptors are G-protein-coupled receptors that are highly expressed throughout the BG (Bradley et al. 1999b,c; Kerner et al. 1997; Kosinski et al. 1998, 1999; Testa et al. 1994, 1998). Behavioral and physiological studies have shown that mGluRs play important roles in regulation of BG function. To date, eight mGluR subtypes (mGluR1-8) have been cloned and are classified into three major groups based on sequence homology, coupling to second-messenger systems, and selectivities for various agonists (Conn and Pin 1997). Group I mGluRs (mGluR1 and -5) couple to G_q and activation of phosphoinositide hydrolysis, while group II mGluRs (mGluR2 and -3) and group III mGluRs (mGluR4 and -6 to -8) couple to $G_{i/o}$ and associated effector systems such as

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adenylyl cyclase. The mGluRs (with the exception of mGluR6) are widely distributed throughout the CNS and play important roles in regulating cell excitability and synaptic transmission at excitatory and inhibitory synapses.

We have previously shown that presynaptically localized group II mGluRs inhibit glutamatergic transmission at the STN-SNr synapse and therefore can reduce pathological conditions of overexcitation of GABAergic SNr neurons, providing a useful approach for the treatment of Parkinson's disease (Bradley et al. 2000). Furthermore we have shown that postsynaptically localized group I mGluRs produce a direct excitation of GABAergic SNr neurons (Marino et al. 1999, 2000). Interestingly, recent immunocytochemical studies reveal that group III mGluRs are also present in the SNr (Bradley et al. 1999b; Kosinski et al. 1999). However, the physiological roles of group III mGluRs in the SNr are not known. We now report that activation of group III mGluRs decreases transmission at inhibitory and excitatory synapses onto nondopaminergic, presumably GABAergic, SNr neurons and that these effects are mediated by presynaptic mechanisms.

METHODS

Materials

Bicuculline, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (RS)- α -cyclopropyl-4-phosphonophenylglycine (CPPG), D(-)-2-amino-5phosphonopentanoic acid (D-AP5), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), and L-serine-O-phosphate (L-SOP) were obtained from Tocris (Ballwin, MO). 2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) propanoic acid (LY341495) was a gift from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). All other materials were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole cell patch-clamp recordings were obtained under visual control as previously described (Bradley et al. 2000; Marino et al. 1998). Fifteen- to 18-day-old Sprague-Dawley rats were used for all patch clamp studies. Some animals were transcardially perfused with an ice-cold sucrose buffer [which contained (in mM) 187 sucrose, 3 KCl, 1.9 MgSO₄, 1.2 KH₂PO₄, 20 glucose, and 26 NaHCO₃ equilibrated with 95% O2-5% CO2]. While this tended to increase slice viability, it did not have any effect on experimental outcome. Therefore data from perfused and nonperfused animals have been pooled. Brains were rapidly removed and submerged in ice-cold sucrose buffer. Parasaggital slices (300-µm thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber containing normal artificial cerebrospinal fluid [ACSF, which contained (in mM) 124 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 2.0 CaCl₂, 20 glucose, and 26 NaHCO₃ equilibrated with 95% O₂-5% CO₂]. In all experiments, 5 μ M glutathione and 500 μ M pyruvate were included in the sucrose buffer and holding chamber. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room-temperature ACSF (~3 ml/min, 23-24°C). Neurons in the substantia nigra pars reticulata were visualized with a $\times 40$ water-immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with (in mM) 140 potassium gluconate, 10 HEPES, 10 NaCl, 0.6 EGTA, 0.2 NaGTP, and 2 MgATP, pH adjusted to 7.4 with 0.5 N KOH. Electrode resistance was 3-7 M Ω . For measurement of synaptically evoked currents, bipolar tungsten electrodes were used to apply stimuli.

Nondopamiergic, presumably GABAergic, SNr neurons were identified according to previously established electrophysiological criteria (Richards et al. 1997). Nondopaminergic neurons exhibited spontaneous repetitive firing, short-duration action potentials, little spike frequency adaptation, and a lack of inward rectification, while dopaminergic neurons displayed no or low-frequency spontaneous firing, longer duration action potentials, strong spike frequency adaptation, and a pronounced inward rectification. All of the data presented in these studies are from neurons that fit the electrophysiological criteria of nondopaminergic neurons.

Measurement of inhibitory and excitatory postsynaptic currents (IPSCs/EPSCs)

IPSCs were evoked with the stimulation electrode placed within the SNr rostrally or caudally to the recorded cell outside the cerebral peduncle and recorded at a holding potential of -50 mV. CNQX (10-20 μ M) and D-AP5 (10-20 μ M) were present in the bath to block excitatory transmission. To study miniature IPSCs (mIPSCs), the 140 mM potassium gluconate in the internal solution was substituted with 140 mM CsCl to reduce postsynaptic mGluR effects and increase current amplitude. Therefore outward mIPSCs were recorded at a holding potential of -80 mV in the presence of 1 μ M tetrodotoxin (TTX).

EPSCs were evoked with the stimulation electrode placed into the STN and recorded from a holding potential of -60 mV. Picrotoxin (50 μ M) was bath applied during all EPSC recordings to block inhibitory transmission. For studies of mEPSCs, slices were bathed in standard ACSF with the addition of mannitol (50 mM), TTX (500 nM), and bicuculline (10 μ M) warmed to 25°C. Miniature EPSCs were recorded from a holding potential of -80 mV. For measurement of kainate-evoked currents kainate (100 μ M) was pressure ejected into the slice from a low-resistance pipette as previously described (Bradley et al. 2000; Marino et al. 1998). Kainate-evoked currents were recorded from a holding potential of -60 mV, and slices were bathed in ACSF containing 500 nM TTX.

RESULTS

Previous studies have shown that group III mGluRs are expressed in the SNr and in nuclei sending major inhibitory and excitatory projections to this structure (Bradley et al. 1999b; Kosinski et al. 1999). We therefore determined whether specific agonists of group III mGluRs have an effect on inhibitory or excitatory transmission in SNr neurons.

Activation of group III mGluRs suppresses inhibitory synaptic transmission (IPSCs) in the SNr

Whole cell patch-clamp recordings were made from electrophysiologically identified nondopaminergic neurons of the SNr in midbrain slices. IPSCs were evoked by stimulating within the SNr with bipolar stimulation electrodes (0.4–12.0 μ A, every 30 s) and were recorded at a holding potential of -50 mV in the presence of AMPA receptor (CNQX; 10–20 μ M) and *N*-methyl-D-aspartate (NMDA) receptor (D-AP5; 10–20 μ M) antagonists to block excitatory synaptic transmission. Bicuculline (10 μ M; n = 8; data not shown) abolished evoked IPSCs in all cells tested, confirming that the evoked currents were GABA_A receptor-mediated responses.

Short (3 min) bath application of the group III mGluR selective agonist L-AP4 (100 μ M) significantly reduced the amplitude of evoked IPSCs by 53.1 ± 4.7% (mean ± SE; Fig. 1*A*; P < 0.05, n = 9). This effect of L-AP4 was reversible (Fig. 1*B*). Most experiments were performed at room temperature because increasing the temperature decreased slice viability.





FIG. 1. Application of L(+)-2-amino-4-phosphonobutvric acid (L-AP4) suppresses inhibitory postsynaptic currents (IPSCs) in substantia nigra pars reticulata. A: example traces of evoked IPSCs before (Pre-Drug), during (L-AP4), and after (Washout) brief bath application of L-AP4. B: average time course of the effect of 100 μ M L-AP4 demonstrating that the effect of L-AP4 on IPSCs is reversible. Each point represents the mean (±SE) of data from 6 cells. C: dose-response relationship of L-AP4-induced suppression of IPSCs. The effect of inhibition of IPSCs shows an EC $_{50}$ of around 20 $\mu M.$ Each point represents the mean $(\pm SE)$ of 4 experiments. The effect of L-AP4 on IPSCs is mediated by group III metabotropic glutamate receptors (mGluRs). D: bar graph showing the average effect of the selective agonists L-AP4 (100 µM) and L-serine-O-phosphate (L-SOP, 1 mM) and the effect of the antagonist (RS)- α cyclopropyl-4-phosphonophenylglycine (CPPG, 500 μ M) on the L-AP4-induced inhibition of IPSCs. Each bar represents the mean (\pm SE) of data collected from 5 cells (*P < 0.01, *t*-test).

However, control experiments performed at 32°C revealed that L-AP4 also reduced IPSCs at higher temperatures (75.6 \pm 10.5% inhibition, n = 4). Concentration response analysis revealed that the inhibition of IPSCs by L-AP4 was concentration dependent. It furthermore suggested a biphasic effect with a small response to concentrations between 1 and 10 μ M and a more robust response at higher concentrations (Fig. 1C).

To further pharmacologically characterize the effect of group III mGluR activation on GABAergic synaptic transmission in the SNr, we determined the effect of another group III mGluR-selective agonist and a group III mGluR-selective antagonist. The reduction of IPSC amplitudes induced by L-AP4 was mimicked by 1 mM L-SOP, another selective agonist for group III mGluRs (Fig. 1D). Furthermore the response to L-AP4 (100 μ M) was completely blocked by a 10- to 15-min preincubation with the group II/III mGluR antagonist CPPG (500 μ M) (Fig. 1D) (Toms et al. 1996). Since we have previously shown that activation of group II mGluRs has no effect

on inhibitory synaptic transmission in the SNr (Bradley et al. 2000), these data are consistent with the hypothesis that this response is mediated by activation of a group III mGluR.

Effect of group III mGluR-selective agonists on IPSC amplitudes is mediated by a presynaptic mechanism

To examine the site of action of group III mGluR-selective agonists, we determined the effect of a maximal concentration of L-AP4 on the amplitude of spontaneous mIPSCs. All mIPSC recordings were preformed at a holding potential of -80 mV in the presence of CNQX (10-20 μ M) and D-AP5 (10-20 μ M) to block glutamatergic synaptic currents and 1 μ M tetrodotoxin to block activity dependent release of transmitter. Miniature IPSCs were measured as inward currents with pipettes in which Cl⁻ (140 mM) was the major anion in the internal solution.

Application of the group III-selective agonist L-AP4 (500 μ M) induced a significant decrease in the frequency of mIPSCs



FIG. 2. Inhibition of IPSCs induced by the activation of group III mGluRs is mediated by a presynaptic mechanism. A: examples of miniature IPSC (mIPSC) traces before (pre-drug) and during application of 500 μ M L-AP4. B: amplitude histograms of mIPSCs before (*left*) and during application of 500 μ M L-AP4 (*right*). C: cumulative probability plots showing the lack of an effect of L-AP4 on mIPSC amplitude (*left*) and a decrease in inter-event interval (*right*). Data shown are pooled data from 4 separate experiments.

(Fig. 2A, P < 0.05, n = 4, t-test) while not affecting mIPSC amplitude (Fig. 2, A and B). Thus L-AP4 induced a rightward shift of the inter-event interval cumulative probability plot but had no effect on the amplitude cumulative probability plot (Fig. 2C). The average mIPSC frequency before drug application was 1.75 ± 0.16 Hz and 1.31 ± 0.06 Hz after application of 500 μ M L-AP4 (P < 0.05; n = 4, t-test). The average mIPSC amplitude was 26.7 ± 4.1 pA before and 27.5 ± 3.3 pA after L-AP4 application (P > 0.05; n = 4, t-test). These findings are consistent with a presynaptic site of action for the group III mGluR-mediated suppression of synaptic transmission. To further test this hypothesis, we also determined the effect of L-AP4 on paired-pulse facilitation of evoked IPSCs. All paired-pulse recordings were made in the presence of CNQX (10-20 µM) and D-AP5 (10-20 µM) with standard internal solution to allow measurement of outward IPSCs. IPSCs were evoked every 30 s by paired stimulations of equal strength with a 50-ms inter-pulse interval. At these intervals paired-pulse facilitation was observed in all recordings (Fig. 3A, $148.4 \pm 5.2\%$, n = 11). Only cells that showed an agonist induced effect on the amplitude of the first IPSC of at least 25% inhibition were used for analysis. Under these conditions L-AP4 (100 μ M) induced an increase in the ratio of pairedpulse facilitation in 9 of 10 cells (Fig. 3). In these 10 cells, the mean potentiation before drug application was 150.3 ± 5.4 and

194.5 \pm 45.6% in the presence of L-AP4 (P < 0.01, n = 10, 2-tailed *t*-test). This represents an increase of paired-pulse facilitation induced by L-AP4 of 28.8 \pm 7.3%. Taken together, these data suggest that L-AP4 reduces transmission at inhibitory synapses in the SNr by actions on presynaptic group III mGluRs, resulting in a reduction of GABA release.

Activation of group III mGluRs inhibits excitatory synaptic transmission (EPSCs) at the STN-SNr synapse

EPSCs were elicited by stimulation of the STN with bipolar stimulating electrodes (0.4–12.0 μ A, every 30 s). All recordings were preformed at a holding potential of -60 mV in the presence of picrotoxin (50 μ M) to block inhibitory synaptic transmission. EPSCs elicited with this protocol had a constant latency and were completely abolished with application of 10 μ M CNQX (n = 10, data not shown), suggesting that the synaptic response was a monosynaptic glutamatergic EPSC.

We have previously shown that activation of presynaptically localized group II mGluRs inhibits excitatory transmission at the STN-SNr synapse (Bradley et al. 2000). We now investigated the roles of group III mGluRs in regulating transmission at this synapse. Brief bath application of the group III mGluR selective agonist L-AP4 (500 μ M) produced a significant de-



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FIG. 3. L-AP4 increases the ratio of paired-pulse facilitation of evoked IPSCs. A and B: example traces of paired-pulse experiments before (A) and during application of 100 µM L-AP4 (B). C: overlay of the predrug trace (---) and a trace during application of L-AP4 scaled to the amplitude of the first IPSC (- - -) is shown. L-AP4 increases the ratio of paired-pulse facilitation in 9 of 10 cells. D: bar graph showing the average effect of L-AP4 on the ratio of paired-pulse facilitation in those 10 cells. Each bar represents the mean $(\pm SE)$ of data collected from 10 cells (*P < 0.01; 2-tailed t-test).

EC₅₀ of around 150 μ M with a maximal effect of 72.9 \pm 3.4%

at a concentration of 500 μ M L-AP4 (n = 6, Fig. 4C). As with

the effect of L-AP4 on IPSCs, L-AP4 induced a similar effect

100 µM

pression of EPSCs in nondopaminergic SNr neurons (Fig. 4A; P < 0.01; n = 6). This effect of L-AP4 was reversible (Fig. 4B). The concentration response curve for L-AP4 revealed an



FIG. 4. Application of L-AP4 suppresses excitatory postsynaptic currents (EPSCs) at the subthalamic nucleus-substantia nigra pars reticulata (STN-SNr) synapse by activation of group III mGluRs. A: example traces of evoked EPSCs before (pre-drug), during (L-AP4), and after (washout) brief bath application of L-AP4. Application of L-AP4 dramatically reduces EPSCs in the SNr. B: average time course of the effect of 500 μ M L-AP4 demonstrating that the effect of L-AP4 on EPSCs is reversible. Each point represents the mean (\pm SE) of data from 3 cells. C: dose-response relationship of the L-AP4-induced inhibition of EPSCs. Each point represents the mean (±SE) of data from 3 to 6 experiments. D: bar graph showing the average effects of group III mGluR selective agonists and the effect of the group II/III mGluR selective antagonist CPPG (500 μ M) on the L-AP4-induced effect on EPSCs. Agonists include L-AP4 (500 μ M) and L-SOP (1 mM). Each bar represents the mean $(\pm SE)$ of data collected from 5 cells (*P < 0.05, t-test). E: concentration-response relationships of the group II/III mGluR selective antagonist 2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) propanoic acid (LY341495). Each point represents the mean (±SE) of data obtained from 3 cells.





when measured at 32°C (78.8 \pm 8.8%, n = 3). L-SOP (1 mM), another selective agonist for group III mGluRs, mimicked the effect of L-AP4 on EPSC amplitudes (Fig. 4D). Furthermore, the response to L-AP4 was blocked by prior application (10-15 min) of the group II/III mGluR antagonist CPPG (500 µM, Fig. 4D) (Toms et al. 1996). To further ensure that the effect of L-AP4 is mediated by activation of group III but not group II mGluRs, we also investigated the concentration response relationship of the antagonist LY341495. This antagonist is selective for group II mGluRs but also blocks group III mGluRs at higher concentrations (Kingston et al. 1998). LY341495 blocked the effect of the group II mGluR selective agonist LY354740 with an IC_{50} value of approximately 30 nM. In contrast the IC_{50} value of LY341495 at blocking the response to L-AP4 was approximately 1 μ M (Fig. 4E). These values are consistent with the potencies of LY341495 at group II and group III mGluRs, respectively. Taken together, these data suggest that activation of group III mGluRs inhibits glutamatergic synaptic transmission at the STN-SNr synapse.

Effect of group III mGluR selective agonists on EPSC amplitudes is mediated by a presynaptic mechanism

To test the hypothesis that group III mGluRs mediate the depression of synaptic transmission at the STN-SNr synapse by a presynaptic mechanism, we recorded spontaneous mEPSCs in the presence of TTX (500 nM) to block activity-dependent release. All recordings were preformed at a holding potential of -80 mV and in the presence of bicuculline (10 μ M) to block GABA_A-mediated synaptic currents.

Application of 500 µM L-AP4 had no significant effect on the amplitude or frequency of mEPSCs in SNr neurons (Fig. 5, A-C). The cumulative probability plot for inter-event intervals revealed that L-AP4 did not reduce mEPSC frequency. The average mEPSC frequency was 4.8 ± 1.5 Hz before drug application and 3.4 \pm 0.9 Hz after drug application (P > 0.05, n = 5, t-test). Likewise, the cumulative probability plot of mEPSC amplitudes (Fig. 5C) revealed that L-AP4 did not reduce mEPSC amplitude. The average mEPSC amplitude was M. WITTMANN, M. J. MARINO, S. R. BRADLEY, AND P. J. CONN



FIG. 6. Activation of group III mGluRs does not alter the sensitivity of postsynaptic glutamate receptors in SNr neurons. A: representative traces of kainate-evoked currents in SNr projection neurons before (pre-drug) and during application of 500 μ M L-AP4. B: time course of the effect of L-AP4 on the amplitude of kainate-evoked currents. C: bar graph showing the mean data demonstrating the lack of effect of group III mGluR activation on kainate-evoked currents. Each bar represents the mean (\pm SE) of data collected from 5 cells (P > 0.05, *t*-test).

8.2 \pm 1.1 pA before drug application and 7.3 \pm 0.7 pA after drug application (P > 0.05, n = 5, *t*-test). To further determine the effect of L-AP4 on postsynaptic AMPA receptors, we investigated the effects of maximal concentrations of L-AP4 on currents elicited by brief (50–500 ms) pressure ejection of the nonselective AMPA/kainate receptor agonist kainic acid (100 μ M) into the slice. Kainate application elicited a robust, stable, inward current in the presence of 500 nM tetrodotoxin in nondopaminergic SNr neurons (Fig. 6.4). Application of 500 μ M L-AP4 had no significant effect on kainate-induced currents (Fig. 6, A-C), suggesting that L-AP4 does not modulate kainate-activated channels in SNr neurons.

The lack of an effect of L-AP4 on mEPSC amplitude and on kainate-evoked currents is consistent with a presynaptic site of action. To further test this hypothesis, we determined the effect of L-AP4 on paired-pulse facilitation of evoked EPSCs. All

paired-pulse recordings were performed at a holding potential of -60 mV in the presence of bicuculline (10 μ M) and EPSCs were evoked by stimulating the cerebral peduncle every 20 s by paired stimulations of equal strength at 20- to 100-ms intervals. Stimulus strength and inter-pulse intervals were adjusted in each experiment so that the second EPSC was always greater in amplitude than the first (paired-pulse facilitation: 130.0 \pm 6.5%, n = 7). L-AP4 (500 μ M) reduced the absolute amplitude of EPSCs but also increased the ratio of paired-pulse facilitation significantly to 268 \pm 35.0% (Fig. 7, P < 0.01, n = 7, *t*-test). This represents a 105.9 \pm 24.5% increase of facilitation induced by L-AP4. Taken together, these data provide strong support for the hypothesis that L-AP4 acts presynaptically to inhibit the evoked release of transmitter from glutamatergic terminals.

DISCUSSION

The data presented in this study show that activation of group III mGluRs reduces GABAergic transmission in the SNr and that this reduction is mediated by a presynaptic mechanism. Furthermore we present evidence that activation of presynaptically localized group III mGluRs inhibits excitatory synaptic transmission at the STN-SNr synapse.

All recordings in this study were from electrophysiologically identified nondopaminergic neurons in the SNr. The firing patterns of the cells included in this study, such as spontaneous repetitive firing, short-duration action potentials, little spike frequency adaptation, and a lack of inward rectification, correspond to firing patterns reported for identified GABAergic SNr neurons in vitro (Richards et al. 1997). Furthermore extracellular and intracellular recording studies in vivo show that the majority (ca 80%) of nondopaminergic cells in the SNr can be activated antidromically by thalamic or tectal stimulation (Grofova et al. 1982; Guyenet and Aghajanian 1978), indicating that the majority of nondopaminergic neurons in the SNr are projection neurons. Thus it is likely that most of the neurons investigated in this study are GABAergic projection neurons, representing the major output neurons of the SNr. However, we cannot exclude the possibility that some of our results were obtained from GABAergic interneurons or other unidentified neuronal classes.





Since it is known that a substantial proportion of inhibitory terminals onto SNr projection neurons arise from the striatum (Smith et al. 1998), it is possible that a significant portion of the L-AP4-induced effect is mediated by activation of group III mGluRs at striatonigral synapses, thereby acting on the direct pathway in the BG circuit. However, effects on other GABAergic synapses cannot be excluded since the GABAergic inputs to the SNr are heterogeneous. SNr projection neurons receive GABAergic inputs not only from the striatum but also from the globus pallidus, neighboring SNr projection neurons, and interneurons (Smith et al. 1998).

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In our pharmacological studies, we show that activation of group III mGluRs decreases GABAergic transmission in the SNr. Our findings that L-AP4 has no effect on mIPSC amplitude and increases the ratio of paired-pulse facilitation provides strong evidence for a presynaptic mechanism. The relative high concentration of L-AP4 required to produce a maximal inhibition of IPSCs suggests that this effect is mediated by mGluR7 (Wu et al. 1998). These findings are in agreement with recent anatomical studies that indicate that the mGluR7 subtype is presynaptically localized to symmetric (inhibitory) synapses in the SNr (Kosinski et al. 1999). Interestingly, immunocytochemistry studies reveal that mGluR7 is presynaptically localized at both striatonigral and striatopallidal synapses (Kosinski et al. 1999) but mGluR4 appears to be more abundant at striatopallidal synapses than at striatonigral synapses (Bradley et al. 1999c). Taken together these data suggest that group III mGluRs may play important roles in the modulation of the BG circuit. While mGluR7 localization indicates this receptor subtype could modulate synaptic transmission in the direct as well as in the indirect pathway, the subtype mGluR4 might more selectively modulate activity in the indirect pathway.

We have previously shown that activation of group II mGluRs inhibits excitatory synaptic transmission at the STN-SNr synapse (Bradley et al. 2000). We now demonstrate that activation of presynaptically localized group III mGluRs also inhibits synaptic transmission at this synapse. These findings are consistent with anatomical data demonstrating the presence of mGluR7 presynaptically localized at this synapse (Bradley et al. 1999a). The presynaptic mechanism of action for L-AP4 at the STN-SNr synapse is suggested by three converging findings. First, L-AP4 has no significant effect on mEPSC amplitude. Second, L-AP4 did not reduce the response to exogenously applied kainic acid. Finally, L-AP4 enhanced paired-pulse facilitation. Taken together with anatomical studies demonstrating presynaptic localization of group III mGluRs on STN terminals (Bradley et al. 1999a), those data provide strong evidence that L-AP4 inhibits synaptic transmission by acting at a presynaptic site.

It is interesting that while L-AP4 reduced both EPSCs and IPSCs by a presynaptic mechanism of action, activation of group III mGluRs had differential effects on the frequencies of mEPSCs and mIPSCs. Thus L-AP4 induced a significant reduction in the frequency of mIPSCs but had no significant effect on the frequency of mEPSCs. This raises the possibility that L-AP4 might reduce excitatory and inhibitory synaptic transmission by different presynaptic mechanisms.

There are a number of potential mechanisms by which a receptor could act presynaptically to reduce action potential dependent release without decreasing the frequency of mEP-SCs. For instance, mEPSCs are thought to be voltage indepen-

dent and therefore should be insensitive to modulation of presynaptic voltage-dependent ion channels. If a receptor reduces transmission by inhibiting a presynaptic voltage-dependent calcium channel or increasing conductance through a voltage-dependent potassium channel rather than having some downstream effect on the release machinery, this may reduce evoked responses without affecting mEPSCs. This effect has been demonstrated at a variety of synapses where agents known to act presynaptically, such as the calcium channel blocker cadmium, abolish evoked EPSCs but have no effect on either the frequency or amplitude of mEPSCs (Doze et al. 1995; Gereau and Conn 1995; Parfitt and Madison 1993; Scanziani et al. 1995). If a receptor regulates synaptic transmission by a mechanism that is downstream from the presynaptic calcium increase, this is more likely to lead to a decrease in mEPSC or mIPSC frequency. The present studies do not provide definitive insight into the precise mechanism by which L-AP4 reduces inhibitory and excitatory transmission in the SNr. However, the differential effects of L-AP4 on mEPSCs and mIPSCs may provide some important clues that could guide future studies.

In summary, these studies demonstrate that group III mGluR subtypes are involved in the modulation of both inhibitory and excitatory synaptic transmission in the SNr. These receptors therefore may provide exciting new targets for the development of pharmacological treatments of disorders that are believed to be caused by a shift in the balance of activity in the direct and the indirect pathway, such as Parkinson's disease, Huntington's disease, and Tourette syndrome. By selectively targeting different mGluR subtypes with specific mGluR agonists or antagonists, it may be possible to restore the balance of activity in the BG circuit.

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Distribution and roles of metabotropic glutamate receptors in the basal ganglia motor circuit: implications for treatment of Parkinson's Disease and related disorders

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Abstract

The basal ganglia (BG) are a set of interconnected subcortical structures that play a critical role in motor control. The BG are thought to control movements by a delicate balance of transmission through two BG circuits that connect the input and output nuclei: the direct and the indirect pathways. The BG are also involved in a number of movement disorders. Most notably, the primary pathophysiological change that gives rise to the motor symptoms of Parkinson's Disease (PD) is the loss of dopaminergic neurons of the substantia nigra pars compacta (SNc) that are involved in modulating function of the striatum and other BG structures. This ultimately results in an increase in activity of the indirect pathway relative to the direct pathway and the hallmark PD symptoms of rigidity, bradykinesia, and akinesia. A great deal of effort has been dedicated to finding treatments for this disease. The current pharmacotherapies are aimed at replacing the missing dopamine, while the current surgical treatments are aimed at reducing transmission through the indirect pathway. Dopamine replacement therapy has proven to be helpful, but is associated with severe side effects that limit treatment and a loss of efficacy with progression of the disease. Recently developed surgical therapies have been highly effective, but are highly invasive, expensive, and assessable to a small minority of patients. For these reasons, new effort has been dedicated to finding pharmacological treatment options that will be effective in reducing transmission through the indirect pathway. Members of the metabotropic glutamate receptor (mGluR) family have emerged as interesting and promising targets for such a treatment. This review will explore the most recent advances in the understanding of mGluR localization and function in the BG motor circuit and the implications of those findings for the potential therapeutic role of mGluR-targeted compounds for PD. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Basal ganglia; Parkinson's Disease; Metabotropic glutamate receptors; Therapeutics; Presynaptic

Abbreviations: BG, basal ganglia; CPCCOEt, 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester; DCG-IV, (2S, 2'R,3'R)-2-(2',3'dicarboxycyclopropyl)glycine; EPN, entopeduncular nucleus; GABA, γ -aminobutyric acid; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; L-DOPA, levodopa; mGluR, metabotropic glutamate receptor; MPEP, methylphenylethynylpyridine; PD, Parkinson's Disease; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; TCA, tricyclic antidepressant; TS, Tourette's Syndrome.

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1. Introduction

The basal ganglia (BG) is a collective term used to refer to a group of interconnected subcortical nuclei that provide a feedback loop to many different areas in the cortex and descending influences to brainstem motor regions. One of the most prominent roles of the BG is regulation of motor function. The most striking evidence for such a role is the movement disorder, Parkinson's Disease (PD). The clinical syndrome that occurs in PD patients is characterized by a disabling motor impairment that includes tremor, rigidity, and bradykinesia. A large number of basic and clinical studies reveal that the primary pathophysiological change giving rise to the symptoms of PD is a loss of substantia nigra dopaminergic neurons that are involved in modulating function of the striatum and other BG structures. In order to understand the dysfunction of PD and related movement disorders, it is essential to gain a clear understanding of the normal circuitry and function of the BG.

2. Circuitry of the basal ganglia motor system

The left panel of Fig. 1 provides a schematic diagram of the major aspects of BG circuitry. While this diagram is highly oversimplified, it provides a useful framework for examining BG function. The main excitatory input to the BG is from the motor cortex. The cortex sends excitatory projections to the striatum, the major input nucleus of the BG. Corticostriatal fibers synapse onto striatal y-aminobutyric acid (GABA)ergic projection neurons termed medium spiny neurons for their distinct morphology. These projection neurons can be subdivided into two functional classes. Approximately one-half of the medium spiny neurons project directly to the BG output nuclei, the globus pallidus internal segment [GPi; the entopeduncular nucleus (EPN) in rats] and the substantia nigra pars reticulata (SNr; see Smith & Bolam, 1990). This is called the direct pathway, and provides direct inhibitory control over the BG output nuclei. The inhibitory control over the GABAergic GPi/SNr output cells provided by the direct pathway ultimately leads to a disinhibition of thalamocortical cells. The other one-half of the striatal projection neurons participate in a multi-synaptic relay, referred to as the indirect pathway. GABAergic medium spiny neurons, giving rise to the indirect pathway, project to and inhibit GABAergic cells of the globus pallidus external segment (GPe). GPe cells normally exert tonic inhibitory control over glutamatergic cells of the subthalamic nucleus (STN), but activation of the GABAergic striatopallidal pathway leads to a disinhibition of the STN. This allows for excitatory transmission between the STN and the BG output nuclei, the GPi/SNr, thus inhibiting thalamocortical cells (Bergman et al., 1990; DeLong, 1990).

The direct and indirect pathways of the BG act as a finetuning mechanism in movement control (Alexander et al., 1986). The balance of transmission through the direct and indirect pathways is tightly regulated by a major modulatory projection from dopaminergic neurons in the substantia nigra pars compacta (SNc). This dopamine input to the striatum regulates the direct and indirect pathways differentially, due to the presence of different postsynaptic dopamine receptors on the two populations of medium spiny neurons. D1 receptors are primarily expressed on medium spiny neurons that project directly to SNr, while D2 receptors are primarily expressed on the medium spiny neurons that constitute the indirect pathway (Gerfen et al., 1990). Because of this differential expression, the release of dopamine in the striatum has a net excitatory effect on the direct pathway, and an inhibitory influence on the indirect pathway.



Fig. 1. A schematic representation of BG circuitry. The left panel represents normal transmission through the normal BG. The right panel represents the imbalanced transmission in the parkinsonian BG. The solid arrows represent inhibitory projections, and the open arrows represent excitatory projections. The thickness of the arrows represents the relative activity of that particular projection. Thal, thalamus.

As mentioned in Section 1, the primary pathological change giving rise to the motor symptoms of PD is the selective death of dopaminergic neurons in the SNc. The loss of this important modulatory input results in a decrease in activity through the direct pathway and an increase in activity through the indirect pathway (Albin et al., 1989; Wichmann & DeLong, 1997). These changes lead to increased inhibition of thalamocortical neurons, which is believed to underlie the hallmark symptoms of the disease: rigidity, bradykinesia, and akinesia. The right panel of Fig. 1 schematically illustrates the activity changes in BG-thalamocortical circuitry that are thought to occur in PD. Interestingly, evidence suggests that opposite changes in transmission through the direct and indirect pathways may occur in some other movement disorders. For instance, Tourette's Syndrome (TS) is a relatively common neuropsychiatric disorder that is characterized by motor and phonic tics that can include sudden repetitive movements, gestures, or utterances. According to current models, TS is associated with an increase in striatal dopamine, or in the dopamine sensitivity of striatal neurons, which has effects that are opposite to those seen in PD patients. Thus, the increase in dopaminergic transmission is thought to lead to an increase in activity through the direct pathway and a corresponding decrease in activity through the indirect pathway (Albin et al., 1989; Leckman et al., 1997). Huntington's Disease is another hyperkinetic disorder that is thought to be due to a selective loss of striatal spiny neurons that give rise to the indirect pathway (Albin et al., 1990; Reiner et al., 1988). Again, this should lead to a decrease in activity through the indirect pathway relative to the direct pathway (Albin et al., 1990; Reiner et al., 1988).

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3. Therapeutic restoration of balance of activity through the direct and indirect pathways

Treatment of PD traditionally has utilized strategies for replacing lost dopamine, thereby restoring the critical dopaminergic modulation of BG function. Levodopa (L-DOPA), the immediate precursor of dopamine, was the first highly effective treatment for PD, and remains the most effective drug for treating the motor manifestations of PD (for an extensive review, see Poewe & Granata, 1997). However, while effective early in treatment for a majority of patients, L-DOPA and other dopamine replacement therapies have a number of serious shortcomings. Within 5 years of beginning treatment, most patients begin to experience motor fluctuations, and the efficacy of L-DOPA becomes unpredictable. In addition, patients begin to develop serious side effects that often limit therapy (Poewe et al., 1986; Poewe & Granata, 1997). Because of this, there has been a major focus on developing novel treatment strategies that are aimed at acting downstream of the lost dopaminergic neurons to restore balance to the direct and indirect pathways. This effort has led to development of highly effective surgical treatments, such as pallidotomy (Baron et al., 1996; Laitinen et al., 1992) or high-frequency stimulation of the SNT (Limousin et al., 1995), that are aimed at reducing activity through the indirect pathway. These treatments have been successful in ameliorating parkinsonian symptoms in a subset of patients. Unfortunately, these surgical approaches are highly invasive, extremely expensive, and remain reserved for patients that can no longer be helped by dopamine replacement therapy.

4. Metabotropic glutamate receptors provide novel therapeutic targets for treatment of movement disorders

In recent years, a novel family of receptors for the amino acid transmitter glutamate have been characterized that couple to effector systems through GTP-binding proteins. These receptors, referred to as mGluRs, are widely distributed throughout the CNS, where they play important roles in regulating cell excitability and synaptic transmission (for an extensive review, see Conn & Pin, 1997). One of the primary functions of mGluRs is a role as presynaptic receptors involved in reducing transmission at glutamatergic synapses. The mGluRs also serve as heteroreceptors involved in reducing GABA release at inhibitory synapses. Finally, postsynaptically localized mGluRs often play an important role in regulating neuronal excitability and in regulating currents through ionotropic glutamate receptor channels. If mGluRs play these roles in BG, then members of this receptor family may provide exciting new targets for drugs that restore the balance between the direct and indirect pathways without the problems associated with dopamimetic therapy, and could be useful for relieving the symptoms of PD and related movement disorders. Interestingly, recent studies suggest that members of this receptor family are differentially distributed in several BG nuclei, where they play an important role in regulating neuronal signaling.

To date, eight mGluR subtypes have been cloned from mammalian brain. These subtypes are classified into three major groups based on sequence homologies, coupling to second messenger systems, and pharmacological profiles. Group I mGluRs, which include mGluR1 and mGluR5, couple primarily to G_q and increases in phosphoinositide hydrolysis. Groups II (mGluR2 and mGluR3) and III mGluRs (mGluR4, 6, 7, and 8) couple to G_i/G_o and inhibition of adenylyl cyclase (for reviews, see Conn & Pin, 1997; Anwyl, 1999). This review will provide a brief survey of studies of mGluR localization and function in the BG motor circuit, and the potential relevance of these findings for treatment of PD and other movement disorders. 4

5. Metabotropic glutamate receptors modulate excitatory input to the basal ganglia

The major excitatory drive to the BG, which originates in the cortex and terminates in the striatum (corticostriatal pathway), is modulated presynaptically by Groups II and III mGluRs. Activation of presynaptic Group III mGluRs on the corticostriatal pathway results in an inhibition of glutamatergic transmission (Calabresi et al., 1992; East et al., 1995; Lombardi et al., 1993; Lovinger & McCool, 1995; Pisani et al., 1997a). This effect is likely mediated by the mGluR4 or 7 subtypes, as they have been identified via electron microscopic immunocytochemistry at asymmetric (excitatory) synapses in the striatum (Fig. 2; Bradley et al., 1999b, 1999c; Kosinski et al., 1999). In addition, Group II-specific agonists have been shown to inhibit transmission at this synapse (Lovinger & McCool, 1995). In other brain regions, Groups II and mGluRs act as presynaptic autoreceptors at glutamatergic synapse. These studies suggest that the mGluRs may play a similar role in modulating corticostriatal excitation (Testa et al., 1998). The Group I mGluRs, mGluR1 and 5, have been identified in striatal medium spiny neurons (Fig. 2; Kerner et al., 1997; Testa et al., 1995, 1998). Activation of Group I mGluRs potentiates N-methyl-D-aspartate receptor currents in striatal neurons (Colwell & Levine, 1994; Morari et al., 1994; Pisani et al., 1997b).

It is difficult to make clear predictions regarding the net effect of agonists and antagonists acting at mGluRs in



Fig. 2. A schematic representation showing the confirmed immunoreactivity of mGluR proteins in the BG motor circuit. Numbers in the nuclei boxes represent postsynaptic localization of those subtypes in that nucleus, whereas numbers in the arrows represent presynaptic localization of those subtypes in that projection. An asterisk indicates that a physiological role has been experimentally defined for that subtype in the location. Solid arrows represent inhibitory projections, and the open arrows represent excitatory projections. Thal, thalamus.

the striatum on transmission through the direct and indirect pathways. This would depend on whether these receptors selectively regulate specific populations of the striatal projection neurons. However, behavioral studies with injection of Group I mGluR agonists combined with measurements of c-fos expression in the STN suggest that activation of Group I mGluRs may selectively increase transmission through the indirect pathway (Kaatz & Albin, 1995; Kearney et al., 1997, 1998; Sacaan et al., 1991, 1992). If so, antagonists of the Group I mGluRs could provide a therapeutic benefit to PD patients by selectively reducing activity through the indirect pathway. Likewise, agonists of these receptors may provide a therapeutic benefit for patients suffering from TS or Huntington's Disease.

6. Metabotropic glutamate receptors modulate dopaminergic transmission from the substantia nigra pars compacta to the striatum

The dopaminergic pathway from the SNc to the striatum is also under modulatory control of all three mGluR groups. All three groups of mGluRs have been shown to depress transmission at glutamatergic synapses in the SNc (Wigmore & Lacey, 1998). These effects are likely presynaptic, and suggest that mGluRs are acting as autoreceptors on glutamatergic afferents from the STN, pedunculopontine nucleus, and cortex. Since glutamate release in the SNc is thought to contribute to the degeneration of the SNc neurons observed in PD, it is possible that a mGluR agonist may have the potential to slow the progression of the disease. However, future studies are necessary to determine the role of STN overactivity in contributing to the dopaminergic cell death and to identify the exact mGluR subtypes involved at this synapse.

In addition to presynaptic inhibitory actions, postsynaptic mGluRs play an excitatory role in the SNc. Direct activation of Group I mGluRs depolarizes dopaminergic cells (Meltzer et al., 1997; Mercuri et al., 1993; Shen & Johnson, 1997). Interestingly, brief application of Group I mGluR agonists induces a biphasic response. The initial inhibitory phase of this response was mediated by Group I receptors and was only observed at low agonist concentration and very short application time. This inhibitory effect has been shown to be mediated by the activation of a Ca²⁺-dependent potassium conductance (Fiorillo & Williams, 1998). Anatomical studies confirm that mGluR1 mRNA and protein is indeed localized postsynaptically in dopaminergic SNc neurons (Fig. 2; Kosinski et al., 1998; Testa et al., 1994).

mGluRs also play a presynaptic regulatory role at the nigrostriatal synapse. (\pm) -1-Aminocyclopentane-trans-1,3-dicarboxylic acid (a nonselective mGluR agonist), as well as (RS)-3,5-dihydroxyphenylglycine (a Group I-specific agonist) and (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV, a Group II-selective agonist), all facilitate dopa-

mine release from nigrostriatal terminals (Bruton et al., 1999; Ohno & Watanabe, 1995; Verma & Moghaddam, 1998). This suggests that cortical glutamate release, in addition to directly exciting striatal output neurons, will actually increase dopamine release and facilitate the dopaminergic-mediated increase in direct pathway activity. This might suggest that Group I mGluR agonists could have therapeutic benefit in PD, increasing neurotransmitter release from the surviving dopaminergic terminals. However, the competing effects in the STN and SNr that are discussed in Section 8.3 would likely counteract this.

7. Metabotropic glutamate receptors modulate transmission through the direct (striatonigral) pathway

Approximately one-half of the medium spiny neurons in the striatum project directly to the output nuclei of the BG, the GPi, and the SNr, constituting the direct pathway. Anatomical studies indicate that the mGluR7 subtype is presynaptically localized to symmetric (inhibitory) synapses in the SNr (Fig. 2; Kosinski et al., 1999). These afferents originate in the striatum. Physiological evidence from our laboratory indicates that activation of Groups I and III mGluRs results in disinhibition of nigral output neurons by decreasing GABAergic inhibitory transmission (Wittmann et al., 1999). This resulting increase in the activity of the SNr projection neurons could increase the inhibitory drive from the GABAergic output nuclei onto thalamocortical neurons. This suggests that Group III mGluR-selective agonists could decrease inhibition of the BG output through the direct pathway, and may provide a novel target for treating hyperkinetic disorders, such as Huntington's Disease and TS.

8. Metabotropic glutamate receptors modulate transmission through the indirect pathway

8.1. Striatopallidal synapse

The striatal enkephalinergic medium spiny neurons that give rise to the indirect pathway project to the GPe in primates (Anderson & Reiner, 1990; Beckstead & Kersey, 1985). The GPe is simply referred to as the GP in rodents, where most of the mGluR studies have been performed. This is the first synapse in the indirect pathway. While no functional studies of mGluRs at this synapse have been performed, anatomical studies have demonstrated the existence of mGluR receptors at both sides of the striatopallidal synapse. The Group III mGluRs, mGluR4 and 7, have been localized to presynaptic striatopallidal terminals using both confocal and electron microscopy (Fig. 2; Bradley et al., 1999b, 1999c; Kosinski et al., 1998). GP neurons express mRNA and protein for mGluR1 and 5 (Fig. 2; Testa et al., 1994). Electron microscopic studies have shown postsynaptic localization of mGluR1 (Testa et al., 1998) and 7 (Bradley et al., 1999b; Kosinski et al., 1998) in rat and mGluR5 in primate (Fig. 2; Hanson & Smith, 1999). The anatomical distribution of mGluRs at the striatopallidal synapse has some interesting, hypothetical functional roles. While the primary neurotransmitter released from striatopallidal terminals is GABA, the GP also receives a small glutamatergic input from the STN (Plenz et al., 1998; Shink & Smith, 1995). Activation of the STN could result in activation of presynaptic mGluRs on striatopallidal terminals, thus inhibiting their release of GABA. This ultimately would result in an increase in GP activity that inhibits STN activity. Additionally, STN excitatory input could directly activate pallidosubthalamic neurons via postsynaptic mGluRs on GP neurons that, in turn, would inhibit STN neurons. This negative feedback loop likely acts as an STN regulator in a normally functioning BG. However, when the indirect pathway is overactive, as it is in PD, the small glutamatergic input to the GP may not be enough to keep STN activity in check.

The localization of mGluRs in the GP has some interesting therapeutic implications. Of particular note, if an increase in activity of striatopallidal neurons is indeed important for the motor dysfunction associated with PD, agonists of mGluR4 and/or 7 could reduce transmission at this synapse and provide a therapeutic benefit. However, while there is wide agreement that increased activity of the STN is critical for PD, there is less agreement on the relative role of GP (Hassani et al., 1996; Levy et al., 1997). Thus, it is not yet certain that reducing inhibition of GP would dramatically reduce the overactivity of STN neurons in parkinsonian animals. Another issue to consider is that if Group III mGluRs are also present on terminals of direct striatal projections to the output nuclei, this would counteract the beneficial effect of Group III agonists in the GP. Consistent with this possibility, immunocytochemistry studies reveal that mGluR7 is evenly distributed at striatopallidal and striatonigral synapses (Kosinski et al., 1999). However, mGluR4 appears to be more abundant in striatopallidal synapses than in striatonigral synapses (Bradley et al., 1999c). Thus, it is conceivable that selective agonists of mGluR4 could selectively reduce transmission through the indirect pathway, while having less effect on the direct pathway. In the future, it will be important to perform detailed physiological and behavioral studies to further evaluate the potential therapeutic value of Group III mGluR agonists in the GP.

8.2. Pallidosubthalamic synapse

In contrast to the striatopallidal synapse, less is known about the anatomical distribution of mGluR subtypes at the pallidosubthalamic synapse. However, recent anatomical and functional studies do suggest an important role of mGluRs in the STN. In situ hybridization studies have shown that the pallidosubthalamic neurons express mGluR1 and 5 mRNA, and subthalamic neurons express mGluR1, 2, 3, and 5 mRNA (Testa et al., 1994). Recently, mGluR1 and 5 (Group I) have been localized to dendrites of subthalamic neurons (Fig. 2; Awad & Conn, 1999a, 1999b; Awad et al., 2000). These receptor proteins were found to be postsynaptic at both symmetric (inhibitory, most likely GABAergic pallidal fibers) and asymmetric (excitatory) synapses.

Physiological studies have demonstrated that activation of Group I mGluRs induces a robust depolarization of STN neurons (Awad & Conn 1999a, 1999b; Awad et al., 2000). Interestingly, this depolarization is mediated primarily by the Group I subtype mGluR5. The mGluR5-specific antagonist methylphenylethynylpyridine (MPEP), but not the mGluR1-specific antagonist 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), blocked the depolarization. A functional role for the postsynaptic mGluR1 receptor proteins has yet to be determined. In addition to directly depolarizing STN neurons, Group I activation also increases the frequency of STN burst firing (Awad & Conn, 1999a, 1999b; Awad et al., 2000; Beurrier et al., 1999). This is particularly interesting, as the switch from single-spike firing to a burst-firing mode is one of the characteristics of parkinsonian states in animal models (Bergman et al., 1994; Hassani et al., 1996; Hollerman & Grace, 1992) and parkinsonian patients (Benazzouz et al., 1996; Rodriguez et al., 1997). If this switch does play a role in the neuropathology of the disease, then Group I antagonists potentially may be therapeutic targets.

8.3. Subthalamonigral synapse

As described in Section 2, the overactivity of the glutamatergic STN projection to the BG output nuclei results in an inhibition of thalamocortical neurons. An extremely effective surgical treatment for PD, high-frequency stimulation of the STN (Limousin et al., 1995), is designed to shut down or diminish excitatory input to the EPN/SNr from the STN. In rodents, the primary BG output nucleus is the SNr. Also, to date, the only study of mGluRs in the EPN (homologous to the primate GPi) has been an in situ hybridization study, and the results closely parallel in situ findings in the STN (Testa et al., 1994). Therefore, we will focus on studies of the STN–SNr synapse.

Neurons in the STN express mRNA for mGluR1, 2, 3, and 5 (Testa et al., 1994). Electron microscopic immunocytochemical studies have identified mGluR2/3 (Bradley et al., 1999a) and 7 (Fig. 2; Bradley et al., 1999b; Kosinski et al., 1998) at presynaptic terminals in the SNr that are making asymmetric (excitatory) synapses onto SNr dendrites. These excitatory terminals presumably originate in the STN. GABAergic projection neurons of the SNr express mRNA for mGluR1, 3, and 5 (Testa et al., 1994). Immunocytochemical studies have demonstrated that mGluR1 and 5 are postsynaptically localized at both asymmetric and symmetric synapses in these neurons (Fig. 2; Marino et al., 1999b; Testa et al., 1998). The localization of mGluR subtypes postsynaptic to inhibitory inputs is an intriguing finding. Future studies will be required to elucidate the role of these postsynaptic receptors at nonglutamatergic synapses.

Several recent physiological studies in our laboratory have shed significant light on the role of mGluRs at the subthalamonigral synapse. Both Group II (Fig. 3) and Group III receptors inhibit glutamatergic transmission at this synapse (Bradley et al., 2000; Marino et al., 1999b). This is consistent with anatomical data demonstrating the presence of mGluR2/3 and 7 presynaptically localized at this synapse (Fig. 2; Bradley et al., 2000). Activation of Group I mGluRs produces a robust direct depolarization of SNr GABAergic neurons (Marino et al., 1999b). This effect appears to be mediated by mGluR1 as the mGluR1selective antagonist CPCCOEt, but not the mGluR5selective antagonist MPEP, blocks it. Therefore, in contrast to the Group I-mediated depolarization in STN that was attributable to mGluR5, the analogous effect in SNr seems solely attributable to mGluR1. Moreover, stimulation of glutamatergic afferents to the SNr at frequencies consistent with the normal firing rate of STN neurons induces an mGluR-mediated slow excitatory postsynaptic potential that is blocked by CPCCOEt (Marino et al., 1999a). These data suggest that mGluR1 may play an important role in tonic regulation of BG output.



Fig. 3. Group II mGluRs modulate transmission at the STN-SNr synapse. (A) Evoked excitatory postsynaptic potentials before (control), during (LY354740), and after (wash out) brief local application of LY354740. Applications of LY354740 dramatically reduce excitatory postsynaptic potentials, and this effect is reversible. The effect of LY354740 is mimicked by other Group II mGluR-selective agonists, and blocked by Group II mGluR-selective antagonists (Bradley et al., 2000). (B) Activation of Group II mGluRs reverses catalepsy in an animal model of PD. The degree of haloperidol-induced catalepsy was measured as latency to first paw movement when the animal was placed on a vertical grid. Haloperidol (1.5 mg/kg i.p.) induces a pronounced catalepsy, which was reversed in a dose-dependent manner by LY354740 (3-30 mg/kg i.p.; *P < 0.05). LY354740 alone had no effect (data not shown). Data shown represent mean ± S.E.M. of data collected from eight animals.

Our findings that mGluR2/3 is presynaptically localized on presumed STN terminals in SNr and that activation of Group II mGluRs inhibits excitatory transmission in the SNr are of particular interest to potential alternative therapeutic approaches for PD. An mGluR agonist may be useful in reducing transmission at the STN-SNr synapse. This receptor subtype is a particularly interesting target, due to its restricted localization in the BG. The only other cells in the BG found to express mGluR2 are the striatal cholinergic interneurons (Testa et al., 1994). Therefore, agonists selective for mGluR2 may be useful in alleviating the overactivity of the indirect pathway, without triggering many undesirable side effects. Consistent with this hypothesis, recent behavioral studies have demonstrated that the systemic injection of a the highly selective Group II agonist LY354740 decreases haloperidol-induced muscle rigidity (Konieczny et al., 1998) and catalepsy (Fig. 3; Bradley et al., 2000) in a rat model of PD. Furthermore, injection of another Group II mGluR agonist, DCG-IV, into the SNr has a similar antiparkinsonian effect (Dawson et al., 2000), suggesting that the effect of a Group II agonist is mediated by reducing transmission in the SNr.

9. Summary of the potential metabotropic glutamate receptor-targeted pharmacotherapies

All of these studies demonstrate that multiple mGluR subtypes may be promising potential targets for pharmacotherapies for PD. Antagonists of Group I mGluRs may be useful in reducing transmission through the indirect pathway. A Group I mGluR antagonist could have antiparkinsonian effects by actions at several different sites in the BG motor circuit, including the striatum, STN, and SNr neurons. A potential drawback to a nonselective Group I mGluR antagonist for treatment of PD is that it would also block the Group I mGluR-mediated increase in dopamine release from the SNc neurons. However, since the other sites of action of a Group I antagonist are downstream from dopamine neurons, this action may not significantly alter the therapeutic effect of a Group I antagonist. A Group III mGluR agonist may also be useful in ameliorating the symptoms of PD. Again, a nonselective Group III mGluR agonist may not be ideal because it would reduce GABAergic transmission at both the striatonigral and striatopallidal synapses. This would reduce transmission through both pathways, thus yielding no net effect. However, mGluR4 appears to be selectively expressed in striatopallidal synapses, and may provide a more specific target for attempting to reduce transmission through the indirect pathway. Finally, Group II mGluR agonists may provide the most promising opportunity to date for designing a mGluRmediated therapy for PD. This type of drug would decrease transmission through the indirect pathway by decreasing glutamatergic transmission at the STN-SNr synapse. The restricted expression pattern of mGluR2 in the BG makes this receptor a particularly interesting target. Additionally, the behavioral studies performed to date using a Group II agonist have demonstrated promising results.

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APPENDIX IX

Activation of Metabotropic Glutamate Receptor 5 Has Direct Excitatory Effects and Potentiates NMDA Receptor Currents in Neurons of the Subthalamic Nucleus

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The subthalamic nucleus (STN) is a key nucleus in the basal ganglia motor circuit that provides the major glutamatergic excitatory input to the basal ganglia output nuclei. The STN plays an important role in normal motor function, as well as in pathological conditions such as Parkinson's disease (PD) and related disorders. Development of a complete understanding of the roles of the STN in motor control and the pathophysiological changes in STN that underlie PD will require a detailed understanding of the mechanisms involved in regulation of excitability of STN neurons. Here, we report that activation of group I metabotropic glutamate receptors (mGluRs) induces a direct excitation of STN neurons that is characterized by depolarization, increased firing frequency, and increased burst-firing activity. In addition, activation of group I mGluRs induces a selective potentiation of NMDA-evoked currents. Immunohistochemical studies at the light and

The basal ganglia (BG) are a set of subcortical nuclei that play a critical role in motor control and are a primary site of pathology in a number of movement disorders, including Parkinson's disease (PD), Tourette's syndrome, and Huntington's disease. Recent studies reveal that a key nucleus in the BG motor circuit, the subthalamic nucleus (STN), plays an especially important role in BG function. The STN is an excitatory glutamatergic nucleus in the BG and provides the major excitatory input to the BG output nuclei, the substantia nigra pars reticulata (SNr) and the internal globus pallidus. Normal motor function requires an intricate balance between excitation of the output nuclei by glutamatergic neurons from the STN and inhibition of the output nuclei by GABAergic projections from the striatum (for review, see Wichmann and DeLong, 1997).

Interestingly, recent studies suggest that the major pathophysiological change that occurs in response to loss of nigrostriatal dopamine neurons in PD patients is an increase in activity of STN neurons. The resultant increase in synaptic excitation of GABAergic projection neurons in the output nuclei leads to a "shutdown" of thalamocortical projections and produces the motor impairment characteristic of PD (DeLong, 1990). Conversely, hyperkinetic disorders such as Huntington's disease (Reiner et al., 1988; Albin et al., 1990) and Tourette's syndrome (Albin et al., 1989; Leckman et al., 1997) are associated with decreases in STN activity. These discoveries have led to a major interest in development of novel strategies to treat these disorders by altering neuroelectron microscopic levels indicate that both subtypes of group I mGluRs (mGluR1a and mGluR5) are localized postsynaptically in the dendrites of STN neurons. Interestingly, pharmacological studies suggest that each of the mGluR-mediated effects is attributable to activation of mGluR5, not mGluR1, despite the presence of both subtypes in STN neurons. These results suggest that mGluR5 may play an important role in the net excitatory drive to the STN from glutamatergic afferents. Furthermore, these studies raise the exciting possibility that selective ligands for mGluR5 may provide a novel approach for the treatment of a variety of movement disorders that involve changes in STN activity.

Key words: metabotropic glutamate receptor; subthalamic nucleus; basal ganglia; Parkinson's disease; burst firing; NMDA receptor; mGluR1; mGluR5

nal STN activity or STN-induced excitation of BG output nuclei. Interestingly, surgical lesions (Bergman et al., 1990; Aziz et al., 1991; Guirdi et al., 1996) and high-frequency stimulation of the STN (Benazzouz et al., 1993; Limousin et al., 1995a,b) are highly effective in treatment of PD. Development of a detailed understanding of the mechanisms involved in regulation of STN activity could lead to development of novel therapeutic agents that alter STN activity without surgical intervention.

Recent studies suggest that metabotropic glutamate receptors (mGluRs) play an important role in regulating excitability of neurons in a wide variety of brain regions, including BG structures (Conn and Pin, 1997). If mGluRs are involved in regulating excitation of STN neurons, this could provide a critical component of regulation of STN activity by glutamatergic afferents. Thus, it will be important to determine whether mGluRs are postsynaptically localized in these neurons and whether activation of mGluRs alters STN activity. To date, eight mGluR subtypes have been cloned from mammalian brain and are classified into three major groups based on sequence homologies, second messenger coupling, and pharmacological profiles (for review, see Conn and Pin, 1997). Group I mGluRs (mGluR1 and mGluR5) couple primarily to G_q , whereas group II (mGluR2 and mGluR3) and group III mGluRs (mGluRs 4, 6, 7, and 8) couple to G_i/G_o . We now report that activation of the group I mGluR mGluR5 has a dramatic excitatory effect and selectively increases NMDA receptor currents in STN neurons.

MATERIALS AND METHODS

Slice preparation for electrophysiology. Experiments were performed in STN neurons from 10- to 14-d-old Sprague Dawley rats. Rats were decapitated, the brains were removed, and a block of tissue containing the STN was isolated on ice. The tissue was mounted and immersed in an oxygenated sucrose-artificial CSF (ACSF) solution containing (in mM): 3 KCl, 19 MgSO₄, 1.2 KH₂PO₄, 2 CaCl₂, 187 sucrose, 20 glucose, 26 NaHCO₃, 0.5 pyruvate, and 0.005 glutathione, equilibrated with 95% O₂ and 5% CO₂, pH 7.4. Saggital slices (300 μ m) were prepared using a manual VibroSlice (Stoelting, Chicago, IL) and then incubated at room temperature in ACSF containing (in mM): 124 NaCl, 2.5 KCl, 1.3 MgSO₄, 1 NaH₂PO₄, 2 CaCl₂, 20 glucose, 26 NaHCO₃, 0.5 pyruvate, and 0.005 glutathione, equilibrated with 95% O₂ and 5% CO₂, pH 7.4. In experiments requiring potassium

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channel blockade the ACSF had the following composition (in mM): 105.4 NaCl, 19.6 NaOAc, 2.5 KCl, 1.3 MgCl₂ 0.1 CaCl₂, 0.1 CdCl₂, 2 BaCl₂, 6 CsCl, 20 glucose, 26 NaHCO₃, 3 4-aminopyridine (4-AP), and 25 tetraethylammonium-Cl.

Electrophysiological recordings. After a 2 hr incubation, the slices were transferred to a recording chamber mounted on the stage of an Olympus Optical (Tokyo, Japan) microscope and continuously perfused at 1–2 ml/min with oxygenated ACSF containing 50 μ M picrotoxin and 0.5 μ M tetrodotoxin (except when firing was studied). Recordings were made with terrodotoxin (except when firing was studied). Recordings were made with visualized patch-clamp techniques using Nomarski optics with a water immersion $40 \times$ objective. Whole-cell patch-clamp recordings were made using patch electrodes pulled from borosilicate glass on a Narishige (To-kyo, Japan) vertical puller. Electrodes were filled with (in mM): 140 potassium gluconate, 10 HEPES, 10 NaCl, 0.2 EGTA, 2 MgATP, and 0.2 NaGTP. Internal solutions used in experiments requiring potassium chan-el block contained 140 mw cesium methylsulforate in place of potsein nel block contained 140 mM cesium methylsulfonate in place of potassium gluconate. Signals were recorded using a Warner PC-501A patch-clamp amplifier (Warner Instrument Corp., Hamden, CT) and a pClamp data acquisition and analysis system (Axon Instruments, Foster City, CA). For measurement of NMDA and kainate-evoked currents, NMDA (100

For measurement of NMDA and kainate-evoked currents, NMDA (100 μ M-1 mM) with glycine (100 μ M) or kainate (100 μ M) was pressure-ejected into the slice from a low-resistance pipette using a Picospritzer (General Valve, Fairfield, NJ) at pressures ranging from 5 to 20 psi and for durations of 50-200 msec. Currents were recorded from a holding potential of -60 mV. Slices were bathed in ACSF containing 0.5 μ M tetrodotoxin to block synaptic transmission. Agonists and antagonists of mGluRs were then applied by bath infusion for 5 min. NMDA receptor (NMDAR) and kainate receptor current amplitude was measured from baseline to peak of the current the current.

the current. Animal perfusion and preparation of tissue for immunohistochemistry. Five male Sprague Dawley rats were deeply anesthetized with ketamine (20 mg/kg) and transcardially perfused with cold, oxygenated Ringer's solution followed by 500 ml of 4% paraformaldehyde and 0.1% glutaral-dehyde in phosphate buffer (PB, 0.1 M), pH 7.4, followed by 300 ml of cold PB. The brain was removed from the skull and stored in PBS (0.01 M), pH 7.4 before builded and the skull and stored in PBS (0.01 M), pH 7.4, before being sliced on a vibrating microtome into 60 μ m transverse sections. These sections were then treated with 1.0% sodium borohydride for 20 min and rinsed in PBS.

Immunohistochemistry. The sections were preincubated at room temperature in a solution containing 10% normal goat serum (NGS), 1.0% bovine serum albumin (BSA), and 0.3% Triton X-100 in PBS for 1 hr. They were then transferred to solutions containing each of four primary antibodies raised against synthetic peptides corresponding to either the C terminus of mGluR1a (Chemicon, Temecula, CÅ) or to residues 1116–1130 of mGluR1a (Dr. Carmelo Romano, Washington University School of Med-icine, St. Louis, MO) or to the C terminus of mGluR5 (Upstate Biotech-nology, Lake Placid, NY; Dr. Carmelo Romano). Antibodies were diluted at 0.5–1.0 mg/ml in a solution containing 1.0% NGS, 1.0% BSA, and 0.3% Triton X-100 in PBS. The tissue was incubated in this solution overnight at room temperature. The sections were rinsed in PBS and incubated for 1 hr at room temperature in a secondary antibody solution containing biotinylated goat-anti-rabbit IgGs (Vector Laboratories, Burlingame, CA) di-luted 1:200 in the primary antibody diluent solution. After rinsing, sections were put in a solution containing 1:100 avidin-biotin-peroxidase complex (Vector). The tissue was then washed in PBS and 0.05 M Tris buffer before being transferred to a solution containing 0.01 M imidazole, 0.0005% hydrogen peroxide, and 0.025% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in Tris for 7–10 min. Sections were then mounted on gelatin-coated slides, dried, and coverslipped with Permount.

For immunohistochemical analysis at the electron microscopic level, the sections were treated with cryoprotectant for 20 min and transferred to a -80° C freezer for an additional 20 min. The sections were then thawed and treated with successively decreasing concentrations of cryoprotectant and finally PBS. The immunocytochemical procedure was the same as that used for studies at the light level, except that Triton X-100 was not used, and the incubation in the primary antibody was performed at 4°C for 48 hr. After DAB revelation, the sections were processed for the electron microscope. They were first washed in 0.1 M PB for 30 min and then post-fixed in 1.0% osmium tetroxide for 20 min. Afterward, they were washed in PB and dehydrated by a series of increasing concentrations of ethanol (50, 70, 90, and 100%). Uranyl acetate (1.0%) was added to the 70% ethanol to and rooms). Stany accelered to the room of the room o

Five blocks (three for mGluR1a and two for mGluR5) were cut from the STN and mounted on resin carriers to allow for the collection of ultrathin sections using an ultramicrotome (Ultracut 2; Leica, Nussloch, Germany). The ultrathin sections were collected on single-slot copper grids, stained The ultrating sections were collected on single-slot copper grids, stained with lead citrate for 5 min to enhance contrast, and examined on a Zeiss (Thornwood, NY) EM-10C electron microscope. Electron micrographs were taken at 10,000 and 31,500× magnifications to characterize the nature of immunoreactive elements in the STN. *Drugs.* All drugs were obtained from Tocris Cookson (Ballwin, MO), except that (+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohy-drate (LY354740) was a gift from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN), methylphenylethynylpyridine (MPEP) and 7-hydroxy-



Group I mGluR-mediated depolarization of STN neurons. A, Figure 1. Representative current-clame traces of membrane potential changes in response to DHPG (100 μ M), LY354740 (100 nM), and L-AP-4 (1 mM) from a holding potential of -60 mV. *B*, Corresponding change in membrane input resistance accompanying the change in membrane potential. *C*, Mean data ± SEM of membrane potential changes, showing a significant depolarization by the group I-selective agonist DHPG (**p < 0.001). *D*, Dose–response curve of DHPG-mediated changes in membrane potential.

iminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) were gifts from R. Kuhn (Novartis, Basel, Switzerland), and (S)-(+)-2-(3'-carboxy-bicyclo[1.1.1]pentyl)-glycine (CBPG) was purchased from Alexis (San Diego, CA). All other materials were obtained from Sigma. Data analysis. Values are expressed as mean \pm SEM. Statistical significance was assessed using Student's t test.

RESULTS

Group I mGluRs mediate depolarization of STN neurons

Previous studies suggest that STN neurons express multiple mGluR subtypes, including receptors belonging to each of the major groups of mGluRs (groups I-III) (Testa et al., 1994, 1998; Bradlev et al., 1998, 1999). We took advantage of highly selective agonists of each of the mGluR groups to determine whether activation of these receptors has effects on membrane properties of STN neurons. Unless otherwise stated, all studies were performed in the presence of tetrodotoxin (TTX; 0.5 μ M) to block action potential firing. The group I mGluR-selective agonist 3,5dihydroxyphenylglycine (DHPG) (Schoepp et al., 1994) (100 μ M) induced a robust depolarization of STN neurons ($17.1 \pm 1.1 \text{ mV}$; p < 0.001; Fig. 1A,C) that was accompanied by an increase in membrane input resistance (Fig. 1B). In voltage-clamp mode, this could be seen as an inward current with an accompanying decrease in membrane conductance (data not shown). DHPG-induced depolarization was seen in 23 of 24 cells, indicating a relatively homogeneous population of neurons. The dose-response relationship for DHPG-induced depolarization of STN neurons revealed an EC_{50} of ~30 μ M (Fig. 1D), which is consistent with the EC₅₀ value of DHPG at activation of group I mGluRs (Schoepp et al., 1994).

In contrast with DHPG, the group II selective mGluR agonist

LY354740 (100 nM) (Monn et al., 1997) had no effect on membrane potential (Fig. 1*A*,*C*) or input resistance (Fig. 1*B*). Likewise, the group III-selective agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP-4) (1 mM) (Conn and Pin, 1997) had no effect on the membrane potential or input resistance of STN neurons when examined in the presence of the NMDA receptor antagonists D-AP-5 (20 μ M) and MK801 (10 μ M) (Fig. 1*A*-*C*). L-AP-4 (1 mM) did induce a slight depolarization of STN neurons when applied in the absence of NMDA receptor antagonists (data not shown). This is consistent with previous reports that L-AP-4 is a weak NMDA receptor agonist (Davies and Watkins, 1982).

In other neuronal populations, activation of group I mGluRs can induce cell depolarization by inhibiting a leak potassium current (Guérineau et al., 1994) or by increasing an inward cation current Crépel et al., 1994; Guérineau et al., 1995; Pozzo Miller et al., 1995). The finding that the DHPG-induced depolarization or inward current in STN neurons is accompanied by a decrease in membrane conductance suggests that this effect is more likely mediated by inhibition of a leak potassium current. To determine whether the DHPG-induced current has a reversal potential consistent with mediation by inhibition of a potassium current, we performed an analysis of the I-V relationship of the DHPGinduced current in voltage-clamp mode. I-V relationships were determined in the presence and absence of 100 μ M DHPG by a series of voltage steps ranging from -120 to -30 mV in increments of 10 mV. I-V plots were determined in a total of five cells. A representative leak-subtracted I-V plot in the presence and absence of DHPG (100 µM) is shown in Figure 2A. A subtraction of the predrug I-V plot from that in the presence of DHPG yielded an I-V plot of the DHPG-induced current alone (Fig. 2B). DHPG induced a net outward current at hyperpolarized potentials (greater than -80 mV) and an inward current at potentials in the range of the resting potential (Fig. 2B). The reversal potential of the DHPG-induced current is approximately -80 mV.

A reversal potential in a hyperpolarized range coupled with the reduction in membrane conductance is consistent with the hypothesis that DHPG-induced membrane depolarization is partially attributable to inhibition of a leak potassium current. However, the reversal potential is somewhat more depolarized than the predicted equilibrium potential for potassium (-105 mV). This, coupled with the nonlinear I-V curve of the DHPG-induced current, suggests that other factors may also play a role in this membrane depolarization. In some other systems, group I mGluR activation can also activate inward cation currents (Crépel et al., 1994; Guérineau et al., 1995; Pozzo Miller et al., 1995). It is possible that a similar effect occurs in STN neurons. To determine whether the DHPGinduced current is solely a potassium current, we performed ion substitution experiments. Voltage-clamp experiments were performed under conditions of potassium channel block by replacement of a K⁺ ion with a Cs⁺ ion and inclusion of 4-AP and tetraethylammonium in the external bathing solution. DHPGinduced inward current amplitude at -60 mV was significantly reduced (10.8 \pm 2.3 pA; n = 6) compared with that in control conditions (36.2 \pm 8.1 pA; n = 4; p < 0.01; Fig. 2E). However, the current was not completely blocked, and the residual current is probably mediated by ions other than potassium. Consistent with this, the leak-subtracted I-V plot in the presence of potassium channel block has a reversal potential of -30 mV and shows that DHPG causes an inward current at potentials more negative than -30 mV and outward current at potentials more positive than -30mV (n = 3; Fig. 2C, D).

The simplest interpretation of the data presented above is that DHPG induces depolarization of STN neurons by actions on postsynaptically localized group I mGluRs. However, it is possible that DHPG acts by inducing release of another neurotransmitter that then depolarizes STN neurons. Because all of the studies presented above were performed in the presence of TTX, it is unlikely that DHPG acts by increasing cell firing and thereby increasing neurotransmitter release. However, this does not rule out the possibility that DHPG directly depolarizes presynaptic



Figure 2. Ionic basis of DHPG-induced current. A, Leak-subtracted I-V plot in normal K⁺ conditions in the presence and absence of DHPG (100 μ M). B, Subtracted I-V plot representing DHPG-induced current alone, showing reversal potential of approximately -80 mV. C, Representative leak-subtracted I-V plot in the presence of potassium channel block and cesium. D, Subtracted I-V plot representing DHPG-induced current alone, showing reversal potential of -30 mV. E, Mean data \pm SEM of DHPG-induced inward current amplitude (picoamperes) in voltage-clamp mode in normal potassium conditions, potassium block and cesium, and in the presence of cadmium (100 μ M) (**p < 0.01).

terminals, which could lead to calcium influx and neurotransmitter release. To rule out this possibility, we performed experiments in the presence of cadmium (100 μ M) in the bathing solution to block Ca⁺ channel activity. Consistent with an effective block of neurotransmitter release, this concentration of cadmium completely eliminated EPSCs elicited in the STN by stimulation of the internal capsule (data not shown). However, the amplitude of the DHPGinduced current was not significantly different than that seen in control (31.1 ± 6.9 pA; n = 4; Fig. 2E).

When measured in the absence of TTX, the DHPG-induced depolarization was accompanied by a dramatic increase in action potential firing (Fig. 3*A*). This is consistent with a recent report that group I mGluR agonists increase extracellular single unit firing of STN neurons (Abbott et al., 1997). This increase in cell firing was completely eliminated by hyperpolarizing current injection to hold the membrane potential at the predrug level (Fig. 3*A*). This suggests that the increase in firing frequency was strictly attributable to the DHPG-induced depolarization rather than being partially mediated by other changes in membrane properties that allow the cells to fire at a higher frequency. There was no effect of DHPG on other membrane properties of the cell, including spike width, spike amplitude, and the shape or amplitude of after-hyperpolarizations (data not shown). However, DHPG did induce an increase in the incidence of burst firing, a property of STN

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Figure 3. Postsynaptic effects of group I mGluR activation in STN neurons. A, Representative current-clamp traces of firing rate before drug application (at -50 mV) and dramatic increase in the presence of DHPG (100 μ M) that is countered by current injection to return membrane potential to the predrug level. B, DHPG-mediated switch to burst-firing mode (from a holding potential of -60 mV), which is countered by hyperpolarizing current injection to maintain membrane potential at the predrug level. C, DHPG-mediated membrane oscillations in the presence of TTX are also countered by hyperpolarizing current injection. Action potentials are truncated in A and B. Scale bars in C also apply to B.

neurons previously described by Beurrier et al. (1999) (Fig. 3B). Oscillatory activity underlying burst firing was not seen in any of 15 cells examined at a resting potential of -60 mV before DHPG treatment but was seen in 7 of 26 cells (~27%) during the DHPG-induced depolarization. In the absence of TTX, oscillatory activity was accompanied by burst firing (Fig. 3B). When studied in the presence of TTX, the DHPG-induced oscillatory activity underlying burst firing was seen, indicating that such activity may not be dependent on synaptic transmission and may be an intrinsic membrane property of STN neurons (Fig. 3C). Furthermore, burst firing was not seen in any cells treated with the group II agonist LY354740 (n = 6) or the group III mGluR agonist L-AP-4 (n = 9) when the cell membrane was held at -60 mV before drug application.

Group I mGluRs potentiate NMDA-evoked currents

The data presented above suggest that activation of group I mGluRs can exert a direct excitatory effect on STN neurons that could contribute to the overall excitatory drive to this important nucleus in the BG motor circuit. In some other brain regions, activation of mGluRs can also potentiate excitatory synaptic responses by potentiating currents through glutamate-gated cation channels. To determine the effects of mGluR agonists on the



Figure 4. Activation of group I mGluRs potentiates NMDA-evoked currents in STN neurons but has no effect on kainate-evoked currents. A, Representative voltage-clamp traces of NMDA-evoked currents in predrug, agonist, and wash conditions. Only the group I-selective agonist caused a reversible potentiation of NMDA-evoked currents. The group II and III agonists had no effect on NMDA-evoked currents. B, Mean data \pm SEM of percent potentiation of NMDA-evoked currents by DHPG over predrug current amplitude. DHPG caused a significant potentiation compared with vehicle (*p < 0.05). C, Mean data \pm SEM of percent predrug kainate-evoked current amplitude showing no difference compared with vehice. D, Mean data \pm SEM of percent potentiation of NMDA-evoked currents by DHPG in normal K⁺ at -60 and -80 mV, cesium and potassium channel block at -60 mV, and in the presence of Cd²⁺ (100 μ M).

responses of STN neurons to activation of ionotropic glutamate receptors, we used pressure-evoked application of constant amounts of NMDA or kainate onto the cell. Stable baseline NMDA- or kainate-evoked currents were obtained before perfusion of the slice with mGluR agonists. Representative NMDA-evoked current traces are shown before, during, and after mGluR agonist application (Fig. 4A). DHPG (100 μ M) caused a reversible potentiation of NMDA-evoked current amplitude (39.1 ± 9.2%; n = 10; p < 0.05; Fig. 4B). In contrast, the group II- and group III-selective mGluR agonists LY354740 and L-AP-4 had no effect

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Figure 5. Immunostaining for mGluR1a in the STN. A, Low-power light micrograph of mGluR1a in the STN. B, C, Highpower electron micrographs of mGluR1aimmunoreactive dendrites (*Den*) that form asymmetric synapses (arrowheads) with unlabeled terminals. Note that the dendrite in B contains vesicles (arrows). D, High-power electron micrograph of mGluR1a-immunoreactive terminal that forms an asymmetric synapse (arrowhead) with an immunoreactive dendrite. CP, Cerebral peduncle. Scale bars: A, 500 μ m; B-D, 0.5 μ m.

on NMDA-evoked currents (Fig. 4A,B). None of the groupselective mGluR agonists had any effects on kainate-evoked currents in STN neurons (Fig. 4C).

One concern that should be considered with studies of modulation of NMDA-evoked currents in brain slices is that it is impossible to obtain complete voltage control of the entire dendritic region of the STN neurons. Thus, it is possible that DHPG-induced potentiation of the NMDA-evoked current is attributable to depolarization of dendritic regions in which we have not achieved adequate voltage control. If so, the DHPG-induced depolarization may relieve the voltage-dependent magnesium block of the NMDA receptor and thereby cause NMDA-evoked currents to be potentiated. To test for this possibility, we performed a series of experiments to determine the effect of DHPG on NMDA-evoked currents under conditions in which the DHPG-induced depolarization is blocked. First, experiments were performed in normal K⁺ concentrations when holding at the reversal potential of the DHPGinduced current (-80 mV). Also, we determined the effect of DHPG in the presence of conditions that block voltage-dependent potassium channels and thereby reduce the DHPG-induced depolarization. Under both conditions, DHPG-induced inward current was either reduced or absent. In contrast, neither manipulation significantly altered DHPG-induced potentiation of NMDA-evoked currents (Fig. 4D). In addition, experiments were performed in the presence of cadmium (100 μ M) to ensure that the response was not caused by calcium-dependent release of another neurotransmitter from presynaptic terminals. As with the studies of DHPG-induced

inward currents, cadmium had no significant effect on DHPGinduced potentiation of NMDA-evoked currents (Fig. 4D).

mGluR1 and mGluR5 are postsynaptically localized in STN neurons

DHPG is an agonist at both mGluR1 and mGluR5, suggesting that either of these mGluR subtypes could mediate the responses described above. We performed immunocytochemical studies with mGluR1a and mGluR5 antibodies at the electron microscopic level to determine whether both of these receptors are localized at postsynaptic sites in STN neurons.

At the light microscopic level, the STN showed strong neuropil labeling for mGluR1a and mGluR5 (Figs. 5A, 6A). In general, the immunoreactivity was found predominantly in dendritic processes, whereas the level of labeling in cell bodies was very low. In sections immunostained for mGluR1a, labeled dendrites were found in the cerebral peduncle (Fig. 5A), which is consistent with previous Golgi studies showing that the dendrites of neurons located along the ventral border of the STN extend ventrally into the cerebral peduncle (Iwahori, 1978; Afsharpour, 1985).

To determine the exact nature of the immunoreactive neuronal elements, we performed further analysis at the electron microscopic level. Both antibodies primarily labeled dendritic processes, which formed symmetric and asymmetric synapses with unlabeled axon terminals (Figs. 5B-D, 6B-D). In general, the labeling was seen throughout the dendrites, instead of being associated selectively with the plasma membrane (Figs. 5B-D, 6B-D). It is worth



Figure 6. Immunostaining for mGluR5 in the STN. A, Low-power light micrograph of mGluR5 immunostaining in the STN. B, Low-power electron micrograph of mGluR5-immunoreactive dendrites (Den). C, High-power electron micrograph of a small mGluR5-immunoreactive dendrite that forms an asymmetric synapse (arrowhead) with the unlabeled terminal. D, Highpower electron micrograph of a large mGluR5-immunoreactive dendrite that forms a symmetric synapse (arrow) with an unlabeled terminal. The open arrowhead points to a puncta adherentia. CP, Cerebral peduncle. Scale bars: A, 500 μ m; B, 1 μ m; C, D, 0.5 μ m.

noting that such a pattern of labeling was also detected for mGluR5 in the SNr using both immunogold and immunoperoxidase techniques (Hubert and Smith, 1999). In addition to dendrites, mGluR1a immunoreactivity was occasionally seen in a few myelinated and unmyelinated axonal segments as well as a few axon terminals (Fig. 5D). Cell bodies displayed light intracytoplasmic labeling with either antibody.

Immunohistochemical studies were repeated with additional anti-mGluR1a (Romano et al., 1996) and anti-mGluR5 (Reid et al., 1995; Romano et al., 1995) antibodies and showed similar results confirming our findings of the postsynaptic localization of both of these receptor subtypes in the STN (data not shown). This is consistent with previous *in situ* hybridization studies (Testa et al., 1994) as well as studies of mGluR localization at the light level (Testa et al., 1998).

Effects of DHPG in STN neurons are mediated by mGluR5

The postsynaptic localization of both mGluR1a and mGluR5 in STN neurons suggests that either or both of these receptors could be involved in mediating the effects of DHPG. We used newly available pharmacological tools that distinguish between these two group I mGluR subtypes to further characterize the group I-mediated effects in STN. Interestingly MPEP (10 μ M), a highly selective noncompetitive antagonist at mGluR5 (Bowes et al., 1999; Gasparini et al., 1999), blocked DHPG-induced membrane depolarization $(4.2 \pm 0.27 \text{ mV}; p < 0.001; n = 3; \text{Fig. 7}A,B)$. In contrast, the mGluR1-selective noncompetitive antagonist CPCCOEt (100 μM) (Annoura et al., 1996; Casabona et al., 1997; Litschig et al., 1999), had no effect on DHPG-mediated depolarization of STN neurons (18.8 \pm 3.0 mV; n = 3; Fig. 7A,B) at concentrations that have been shown to be effective at blocking mGluR1a in recombinant (Litschig et al., 1999) and native (Casabona et al., 1997) systems. These data suggest that DHPG-induced depolarization of STN neurons is mediated by mGluR5 rather than mGluR1. Consistent with this, CBPG (100 μ M), a partial agonist of mGluR5 with mGluR1 antagonist activity (Mannaioni et al., 1999), mimics DHPG-induced depolarization of STN neurons. As with DHPG, the response to CBPG is blocked by MPEP but not by CPCCOEt (Fig. 7B).

Pharmacological analysis of mGluR-mediated potentiation of NMDA receptor currents suggests that this response is also mediated by mGluR5. As with the depolarization, MPEP blocks



Figure 7. mGluR5 mediates group I mGluR-evoked depolarization of STN neurons. A, Membrane potential traces showing depolarization with DHPG (100 μ M), which is blocked by the mGluR5-selective antagonist MPEP (10 μ M). Membrane depolarization is not blocked by the mGluR1-selective antagonist CPCCOEt (100 μ M). B, Mean data \pm SEM of change in membrane potential showing a significant inhibition of DHPG-mediated depolarization of STN neurons by MPEP (10 μ M) compared with DHPG alone (**p < 0.001). MPEP also significantly blocks depolarization mediated by the mGluR5-selective agonist CBPG (100 μ M) (*p < 0.05).

DHPG-induced potentiation of NMDA-evoked currents $(2.3 \pm 3.2\%; p < 0.05; n = 5)$, whereas CPCCOEt is without effect on this potentiation (44.2 ± 18.9%; n = 6; Fig. 8 *A*,*B*).

DISCUSSION

The data presented reveal that group I mGluRs are postsynaptically localized on neurons in the STN and that activation of these receptors leads to a direct depolarization of STN neurons. In most cells, the DHPG-induced depolarization was accompanied by an increase in firing frequency with no obvious effects on the recently characterized stable oscillations of STN neuronal firing (Bevan and Wilson, 1999). However, in approximately one-third of the cells examined, DHPG induced a switch in the firing pattern from the characteristic single-spike firing mode to a burst-firing mode that was recently characterized in detail by Beurrier et al. (1999). In addition, DHPG induced a selective increase in NMDA receptor currents in STN neurons. These combined effects of group I mGluR activation could provide an important component of the net excitatory drive elicited by activity of the major excitatory afferents to the STN from the cortex or thalamus. However, it is important to note that activation of group I mGluRs has also been shown to induce presynaptic effects on glutamate release in some brain regions (Gereau and Conn, 1995; Manzoni and Bockaert, 1995; Rodriguez-Moreno et al., 1998). If group I mGluRs have similar effects in the STN, the net effect of group I mGluR activation in this region will ultimately depend on a combination of presynaptic and postsynaptic effects.

Immunohistochemistry studies revealed that both mGluR1a and mGluR5 are postsynaptically localized in STN neurons. Interestingly, pharmacological analysis suggested that each of the responses studied was mediated by mGluR5, with little or no contri-



Figure 8. mGluR5 mediates group I mGluR-induced potentiation of NMDA-evoked currents. A, Current traces of NMDA-evoked currents before, during, and after application of DHPG (100 μ M). The potentiation is blocked by MPEP (10 μ M) but not CPCCOEt (100 μ M). B, Mean data \pm SEM of percent potentiation of NMDA-evoked currents by DHPG over predrug conditions. MPEP (10 μ M) significantly blocks potentiation of NMDA-evoked current compared with DHPG alone (*p < 0.05).

bution of mGluR1. This finding suggests that although STN neurons contain both group I mGluR subtypes, there is a segregation of function of these two receptors. It is possible that mGluR1 also plays important roles in regulating STN functions that were not measured in the present studies. Future studies of the roles of mGluR1 in these cells may shed important light on the functions of expression of multiple subtypes of closely related receptors by a single neuronal population.

Implications of mGluR5 actions for treatment of PD

One of the most interesting implications of the finding that mGluR5 is involved in regulating activity and NMDA receptor currents in STN neurons is the possibility that antagonists of this receptor could provide novel therapeutic agents that could be useful for treatment of PD. Traditional dopamine replacement strategies for PD treatment tend to lose efficacy over time, and patients begin to experience serious adverse effects, including motor fluctuations (Poewe and Granata, 1997). Because of this, a great deal of effort has been focused on developing a detailed understanding of the circuitry and function of the BG in the hopes of developing novel therapeutic approaches for the treatment of PD. Interestingly, a large number of animal and clinical studies reveal that loss of nigrostriatal dopamine neurons results in an increase in activity of the STN and that an increase in STN-induced excitation of the output nuclei is ultimately responsible for the motor symptoms of PD (for review, see DeLong, 1990; Wichmann and De-Long, 1997). These findings suggest that pharmacological agents that reduce the excitatory drive to the STN or otherwise reduce STN activity could provide a therapeutic effect in PD patients.

The data reported here suggest that mGluR5 may be a particularly interesting candidate as a receptor that could regulate STN output. Of particular interest is the finding that mGluR5 activation increases burst firing of STN neurons. For instance, previous studies suggest that a transition of STN neurons from single-spike activity to a burst-firing mode is one of the characteristics of parkinsonian states in rats and nonhuman primates (Hollerman and Grace, 1992; Bergman et al., 1994; Hassani et al., 1996) as well as parkinsonian patients (Benazzouz et al., 1996; Rodriguez et al., 1997). The finding that membrane oscillations underlying burst firing occur in the presence of TTX is consistent with the findings of Beurrier et al. (1999) and suggests that burst firing is, in part, an intrinsic property of STN neurons. However, these data do not rule out the possibility that synaptic mechanisms also participate in induction of burst firing (Plenz and Kitai, 1999). Consistent with the hypothesis that group I mGluRs can increase the output of STN in vivo, Kaatz and Albin (1995) recently reported that injection of group I mGluR agonists into the STN induces rotational behavior. Furthermore, Kronthaler and Schmidt reported that (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1996) and (25,35,45)- α -carboxycyclopropyl glycine (1998), agonists of both group I and group II mGluRs, induce catalepsy in rats. These effects are not mimicked by a highly selective agonist of group II mGluRs (Marino et al., 1999b; Bradley et al., 2000), suggesting that it is likely mediated by mGluRs belonging to group I. Taken together, these findings raise the exciting possibility that mGluR5 antagonists could reduce STN activity and thereby provide a therapeutic benefit to PD patients.

For antagonists of group I mGluRs to be effective in the treatment of PD, mGluR5 must be physiologically activated by endogenous glutamate release onto STN neurons from various glutamatergic afferents. We made several attempts to elicit slow mGluR-mediated synaptic responses using single-pulse stimuli as well as stimulus trains of varying frequencies and durations. Unfortunately, we were unable to reliably elicit mGluR-mediated EPSPs or EPSCs in our slices (our unpublished findings). Although disappointing, this is not surprising, because mGluR-mediated slow EPSPs have also been difficult to measure in other brain regions, except those in which there is a laminar or other organization in which glutamatergic afferent pathways are not severed by slice preparation. In addition to severing afferent projections during slice preparation, mGluR-mediated slow EPSPs are often difficult to measure because of the small size of the events as well as the distance between the recording site in the soma and the distal dendrites where the slow EPSP is likely generated. Because of this, failure to measure a slow EPSP does not imply that mGluRs are not synaptically activated in vivo. Also, we often elicited slow EPSPs that were not blocked by antagonists of known receptors and could have occluded an mGluR-mediated slow EPSP (our unpublished findings). Ultimately, the question of whether mGluR5 in the STN is activated by endogenous glutamate may require in vivo electrophysiological and/or behavioral studies in which mGluR5 antagonists are injected into this structure.

Potential therapeutic effects of mGluR5 agonist actions in the STN

In addition to the potential utility of mGluR5 antagonists in treatment of PD, it is important to point out that the actions of mGluR5 agonists could provide a therapeutic benefit in some other motor disorders, such as Tourette's syndrome and Huntington's disease. Tourette's syndrome is a relatively common neuropsychiatric disorder that is characterized by motor and phonic tics that can include sudden repetitive movements, gestures, or utterances. According to current models, Tourette's syndrome is associated with an increase in striatal dopamine or in the dopamine sensitivity of striatal neurons that has effects that are opposite of those seen in PD patients (Albin et al., 1989; Leckman et al., 1997). Huntington's disease is another hyperkinetic disorder that is thought to be caused by a selective loss of striatal spiny neurons that gives rise to the indirect pathway and, consequently, a decrease in STN activity (Reiner et al., 1988; Albin et al., 1990). On the basis of this, it is possible that selective mGluR5 agonists could provide a therapeutic benefit to patients suffering from these hyperkinetic disorders by increasing activity of STN neurons.

Roles of group I mGluRs in other basal ganglia nuclei

Interestingly, agonists of group I mGluRs have actions in other areas of the basal ganglia motor circuit that could complement their actions in the STN. For instance, group I mGluRs, and especially mGluR5, are heavily localized in the striatum (Shigemoto et al., 1993; Tallaksen-Greene et al., 1998), where agonists of these receptors induce excitatory effects similar to those described here in the STN (Calabresi et al., 1993; Colwell and Levine, 1994; Pisani et al., 1997). Furthermore, recent behavioral studies reveal that injection of group I mGluR agonists into the striatum induces turning behavior that is accompanied by an increase in activity of neurons in the STN and BG output nuclei (Sacaan et al., 1991, 1992; Kaatz and Albin, 1995; Kearney et al., 1997). Group I mGluRs are also present in the SNr (Hubert and Smith, 1999). Recent physiological studies from our laboratory suggest that activation of these receptors has direct excitatory effects and decreases evoked IPSCs (Marino et al., 1999a) in SNr neurons. Taken together, these data suggest that group I mGluRs function at multiple levels of the BG circuit to lead to a net increase in activity of neurons in the output nuclei. Thus, in addition to the STN, both antagonists and agonists of group I mGluRs could act at the levels of the striatum and SNr to provide a therapeutic benefit in the treatment of PD or hyperkinetic disorders, respectively.

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Immunohistochemical Localization of Subtype 4a Metabotropic Glutamate Receptors in the Rat and Mouse Basal Ganglia

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ABSTRACT

Recent studies suggest that metabotropic glutamate receptors (mGluRs) may play a significant role in regulating basal ganglia functions. In this study, we investigated the localization of mGluR4a protein in the mouse and rat basal ganglia. Polyclonal antibodies that specifically react with the metabotropic glutamate receptor subtype mGluR4a were produced and characterized by Western blot analysis. These antibodies recognized a native protein in wild-type mouse brain with a molecular weight similar to the molecular weight of the band from a mGluR4a-transfected cell line. The immunoreactivity was absent in brains of knockout mice deficient in mGluR4. mGluR4a immunoreactivity was most intense in the molecular layer of the cerebellum. We also found a striking mGluR4a immunoreactivity in globus pallidus, and moderate staining in substantia nigra pars reticulata and entopeduncular nucleus. Moderate to low mGluR4a immunoreactivity was present in striatum and other brain regions, including hippocampus, neocortex, and thalamus. Double labeling with mGluR4a antibodies and antibodies to either a dendritic marker or a marker of presynaptic terminals suggest a localization of mGluR4a on presynaptic terminals. Immunocytochemistry at electron microscopy level confirmed these results, revealing that in the globus pallidus, mGluR4a is mainly localized in presynaptic sites in axonal elements. Finally, quinolinic acid lesion of striatal projection neurons decreased mGluR4a immunoreactivity in globus pallidus, suggesting a localization of mGluR4a on striatopallidal terminals. These data support the hypothesis that mGluR4a serves as a presynaptic heteroreceptor in the globus pallidus, where it may play an important role in regulating g-amino-n-butyric acid (GABA) release from striatopallidal terminals. J. Comp. Neurol. 407:33-46, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: globus pallidus; corticostriatal pathway; presynaptic heteroreceptor; electron microscopy; confocal microscopy

Parkinson's disease (PD) is a common basal ganglia (BG) neurodegenerative disorder resulting in disabling motor impairment (tremor, rigidity, and bradykinesia). Loss of nigrostriatal dopamine neurons results in a series of neurophysiological changes that lead to overactivity of the globus pallidus (GP: one of the output nuclei of the BG) and consequent "shutdown" of thalamocortical structures to produce the motor symptoms (Ciliax et al., 1997; Wichmann and Delong, 1997). Although therapies have traditionally utilized dopamine replacement strategies, this

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approach eventually fails in most patients. Exciting advances in understanding of the pathological changes in BG circuitry in PD patients coupled with recent findings suggesting abundant localization of metabotropic glutamate receptors (mGluRs) in key BG nuclei suggest that mGluRs could provide novel targets for therapeutic agents designed to treat PD patients. In the present study, we examined the localization of a mGluR subtype protein, mGluR4a, at crucial sites within BG circuits.

Glutamate is the principal excitatory neurotransmitter in the brain, and is present at many synapses along the BG circuits. It is now clear that the physiological effects of glutamate are mediated by ligand-gated cation channels, known as ionotropic glutamate receptors (iGluRs), and by G-protein-linked receptors, referred to as mGluRs. By activating mGluRs, glutamate can modulate transmission and neuronal excitability at the same synapses at which it elicits fast excitatory synaptic responses (see Conn et al., 1995; Conn and Pin, 1997, for reviews). To date, eight mGluR subtypes have been identified by molecular cloning and these receptors can be placed into three groups based on sequence homology, coupling to second messenger systems, and pharmacological profiles (see Conn and Pin, 1997, for review). Group I mGluRs include mGluR1 and mGluR5, which couple primarily to increases in phosphoinositide hydrolysis in expression systems. The group II mGluRs (mGluR2 and mGluR3), and the group III mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8), all couple to inhibition of cAMP production in expression systems.

Previous studies suggest that presynaptic group II and group III mGluRs play important roles in regulating excitatory (Lovinger, 1991; Lovinger et al., 1993; Lovinger and McCool, 1995; Pisani et al., 1997) and inhibitory (Calabresi et al., 1992, 1993; Stefani et al., 1994) transmission in the striatum (STR) and that postsynaptic group I mGluRs regulate striatal cell excitability and N-methyl-Daspartic acid (NMDA) receptor currents (Calabresi et al., 1992; Colwell and Levine, 1994; Pisani et al., 1997). Moreover, intrastriatal injection of mGluR agonists induces rotational behavior (Sacaan et al., 1991, 1992; Kaatz and Albin, 1995; Kearney et al., 1997) and this effect is abolished by lesions of the subthalamic nucleus (Kaatz and Albin, 1995).

Despite these advances in our understanding of the roles of mGluRs in STR, little is known about the roles of mGluRs in regulating the function of other BG structures. However, in recent years, we and others have used in situ hybridization and immunocytochemistry techniques to show that specific mGluR subtypes are richly distributed in BG structures where they are differentially localized at specific pre- and postsynaptic sites. For instance, group I mGluRs, mGluR1 and mGluR5, are expressed in the substantia nigra pars reticulata (SNpr) and entopeduncular nucleus (ET) neurons (Testa et al., 1994) and mGluR1a immunoreactivity is abundant along the surface of microtubule-associated protein 2 (MAP2)-immunoreactive dendritic processes in GP and SNpr (Testa et al., 1998). Furthermore, in situ hybridization studies suggest that group II mGluR subtype mGluR2 is moderately abundantly expressed in neurons of the subthalamic nucleus (STN; Testa et al., 1994). Finally, both mGluR4 and mGluR7 (but not mGluR6 or mGluR8) mRNA are moderately abundantly expressed in STR (Nakajima et al., 1993; Testa et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995; Kosinski et al., 1999) and double-labeling in situ hybridization revealed that mGluR7 mRNA is in projec-

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tion neurons (Kosinski et al., 1999). More recent immunocytochemistry studies show that mGluR7a is widely distributed in rat brain (Bradley et al., 1996, 1998; Shigemoto et al., 1996, 1997) and it is presynaptically localized on terminals of g-amino-n-butyric acid (GABA)ergic striatopallidal and striatonigral synapses (Kosinski et al., 1999).

The finding that mGluR4 is expressed in striatal neurons suggests that this mGluR could play an important role in regulating BG function. However, the precise localization of mGluR4 protein in BG is not known. To begin to dissect the distribution of the mGluR4a in BG, we have produced and characterized polyclonal antibodies highly specific for mGluR4a.

MATERIALS AND METHODS Production and characterization of polyclonal antibodies that specifically interact with mGluR4a.

Antibodies were generated against synthetic peptides corresponding to the putative intracellular C-terminal domain of mGluR4a. Rabbit polyclonal antisera were prepared and affinity-purified as described previously (Bradley et al., 1996). These antibodies specifically recognized mGluR4a but not other mGluR subtypes or the other mGluR4 splice variant, mGluR4b, as shown below. In double labeling experiments, incubations were performed using combination of mGluR4a antibodies with one of the following: monoclonal antibodies to MAP2 (Sigma, St. Louis, MO; 1:1,000), Enkephalin (Enk; Chemicon Inc., Temecula, CA; 1:2,000); synaptic vesicle protein 2 (SV2; 1:10) obtained from Dr. K. Buckley, Harvard Medical School (Buckley and Kelly, 1985).

Immunoblot analysis

Spodoptera frugiperda, Sf9, insect cells transfected with mGluR4a or mGluR4b were generously supplied by Dr. David Hampson (University of Toronto, Canada). BHK cells stable transfected with mGluR1a were generously supplied by Betty Haldeman of Zymogenetics (Seattle, WA). BHK cells stable transfected with mGluR7a (Saugstad et al., 1994) were generously supplied by Dr. Thomas Segerson (Vollum Institute, Portland, OR). BHK cells transfected with mGluR2 were generously supplied by Dr. Christian Thomsen (Novo Nordisk), and HEK cells transfected with mGluR5 were kindly supplied by Dr. Carl Romano (Washington University School of Medicine, St. Louis, MO). Membranes prepared from transfected cells and from rat brain regions were used for immunoblot studies with the mGluR-directed antibodies. Adult male Sprague-Dawley rats were killed and microdissected brain regions were immediately homogenized with a Brinkman Polytron in 10 mM Tris and 1 mM EDTA (pH 7.4) containing 2 µg/ml of leupeptin, 2 µg/ml of aprotinin, 2 µg/ml of pepstatin A. The homogenates were centrifuged at 2,250 RPM for 5 minutes, the supernatants decanted and recentrifuged at 13,000 RPM for 30 minutes. The membrane pellets were resuspended, protein concentration determined, and membranes kept at -70°C until use. Membranes from rat brain or cell lines were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide) and transferred to PVDF membranes (Millipore, Bedford, MA) by electroblotting as described by Towbin et al. (1979). The blots were blocked

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with 5% nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris-HCl in 1.5% NaCl; pH 7.4) at room temperature for 30 minutes. Blots were then incubated overnight at 4°C with affinity-purified mGluR antibodies (0.5 mg/ml) in TBS. Membranes were then rinsed and incubated for 30 minutes with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) at room temperature. Following several washes in TBS, immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc, Piscataway, NJ), as recommended by the manufacturer. For preadsorption experiments, antibodies were preadsorbed with 10 µg/ml of homologous peptide for 1 hour at room temperature.

Immunocytochemistry

Immunohistochemistry was performed using well established methods (Bradley et al., 1996). For examination at the light microscopic level, adult male Sprague-Dawley rats (n = 10) were anesthetized with 4% chloral hydrate and transcardially perfused with 3% paraformaldehyde followed by 10% sucrose (200-250 ml of each). The protocol used in these studies has been reviewed and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Rat brains were removed and cryoprotected in 30% sucrose at 4°C in 0.1 M phosphate buffer (pH 7.6; 48 hours at 4°C), frozen on dry ice, and sectioned at 40-50 µM on a freezing sliding microtome. Mouse brains (n = 6) were rapidly removed and fixed by immersion in 3% paraformaldehyde for 7 hours at 4°C, then incubated in 10% sucrose for 48 hours at 4°C and finally incubated in 30% sucrose for 48 hours at 4°C. Mouse brains were then frozen on dry ice and sectioned at 40-50 µM on a freezing sliding microtome. Sections were collected in 50 mM TBS (pH 7.2) at 4°C. Tissue sections through the entire brain were processed for immunocytochemistry. Sections were preblocked in TBS with 4% normal goat serum (NGS) and avidin (10 mg/ml) for 30 minutes and subsequently in TBS with 4% NGS and biotin (50 µg/ml) for another 30 minutes. The sections were then incubated with primary antibody (0.5 µg/ml) in TBS and 2% NGS over two nights at 4°C. The avidin-biotin peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used to detect mGluR4a immunoreactivity. The tissue was rinsed several times in TBS and the peroxidase reaction was developed in 0.05% DAB and 0.01% H₂O₂ for 10 minutes. Sections were finally rinsed in TBS and mounted on subbed slides. Sections were dehydrated in alcohols, defatted in xylene, and coverslipped for analysis.

Control experiments were performed in which sections were incubated in TBS without primary antibody. Further control experiments were performed in which primary antibody was preincubated for 30 minutes at room temperature with the homologous peptide (10 mg/ml).

For electron microscopy, Sprague-Dawley rats 250–300 g (n = 4) were deeply anesthetized with 4% chloral hydrate, then perfused transcardially with a phosphatebuffered solution of 3% paraformaldehyde and 0.1 % glutaraldehyde for 10 minutes (250 ml). Brains were postfixed for 1 hour at 4°C and then and sectioned at 40 μ m using a vibrotome (Technical Products International, Inc., St. Louis, MO). Sections were collected in 0.1 M phosphate buffer then rinsed several times with TBS (10 minutes each rinse) before being processed as described for light microscopy. Following the treatment with 0.05% DAB and 0.01% H₂O₂ for 10–15 minutes, the sections were rinsed several times in TBS. Sections were then incubated overnight in 2% glutaraldehyde in 0.1 M phosphate buffer. After rinsing the sections twice for 10 minutes. in phosphate buffer and then in cacodylate buffer (0.1 M), they were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes. Slices were then rinsed twice for 10 minutes in 0.1 M cacodylate buffer, followed by a rinse in 0.05 M acetate buffer. Slices were block-stained overnight in aqueous 2% uranyl acetate, followed by a rinse in 0.05 M acetate buffer. The tissue was then dehydrated in graded ethanols and finally in propylene oxide for 5 minutes before leaving overnight in Epon 1:1 propylene oxide. The

sections were finally embedded in Epon resin between glass slides and left at 60°C for 2 days. Blocks were dissected from the GP and mounted on stubs and sectioned using an ultramicrotome (RMC MT5000 or Reichert Ultracut). Ultrathin sections were collected on uncoated copper mesh grids for analysis with electron microscopy (H-7500, Hitachi).

Fluorescence double-label immunohistochemistry

Dual-label immunohistochemistry was conducted as described in Kosinski et al. (1997). Sections from Sprague-Dawley rat brains (n = 5) were washed in 0.1 M sodium phosphate buffer/saline, pH 7.4, containing 0.9% NaCl, incubated in 3% normal goat serum with 0.3% Triton X-100 in sodium phosphate buffer/saline, pH 7.4, and 0.1% sodium azide for 1 hour, and then incubated 48 hours at 4°C in a solution containing the primary antibodies to mGluR4a in combination with one of the monoclonal antibodies. Sections were then washed in phosphatebuffered saline (PBS) and incubated sequentially in two fluorescent secondary antibodies: the mGluR4a antibodies was visualized using a goat anti-rabbit antiserum coupled to indocarbocyanide (Cy3; 1:400; Jackson Labs., West Grove, PA) whereas Enk, the presynaptic marker SV2, and MAP2 staining were visualized with goat anti-mouse antiserum labeled with fluorescein isothiocynate (FITC) or Cy5 (both from Jackson Laboratories). The sections were then mounted onto gelatin-coated slides, dried, and coverslipped by using glycerol containing 100 mM Tris, pH 8.0, and 0.2% p-phenylenediamine (Sigma, St. Louis, MO) to retard fading. Each experiment included control tissue, processed with omission of one or both primary antibodies.

Preparations were examined using a BioRad Laser Confocal System (MRC 1000) equipped with a Leica DMBR microscope and an argon-krypton laser. High magnification images were obtained by illuminating the section with a single laser line and collecting the image using an appropriate emission filter: for Cy3, excitation at 488 nm and a 522 nm bandpass filter. For each wavelength, four sequential images 1024×1024 pixels in size with an 8-bit pixel depth were obtained and averaged, using a Kalman filtering method to reduce noise. Dual label images were obtained by collecting the separate images sequentially, and reconstructing the images in color using Adobe Photoshop software (Mountain View, CA).

Striatal quinolinic acid lesion

Quinolinic acid (100 nmol in 2 ml 0.1 M phosphate buffer, pH 7.4, Sigma) was injected into the left anterior striatum of adult male Sprague-Dawley rats (Charles River; 200–250 g; n = 3) as described previously (Orlando et al., 1995). Animals were anesthetized with sodium



Fig. 1. Molecular specificity of metabotropic glutamate receptor (mGluR4a) antibodies. Western blot analysis was used with affinitypurifed polyclonal antibodies directed against mGluR4a from different rabbits to determine immunospecificity in membranes from wild type mouse (+/+) cerebellum and cortex, from mouse lacking mGluR4 (-/-; same regions), and from rat cerebellum. The left panel shows immuno-

reactivity with mGluR4a-directed antibodies that do not react with any proteins in mutant mouse, wheras the right panel shows a different elution of antibodies that recognize a protein in mutant mouse with identical molecular weight than mGluR4a. Molecular weight standards are designated on left in kilodaltons (kDa).

pentobarbital (Anpro Pharmaceutical) and surgery was performed in a stereotaxic apparatus using coordinates 2.6 mm lateral to and at the anteroposterior position of bregma, and 4.5 mm ventral to the dural surface (Paxinos and Watson, 1982). Injections were made over 3 minutes with a 10-ml Hamilton syringe fitted with a 26-gauge blunt-tipped needle. Transcardiac perfusion was performed 2 weeks after lesioning as described above. The extent of the lesions were evaluated on thionin-stained coronal sections through the striatum. Three brains in which the lesion was restricted to the anterior striatum and no damage was detected in the GP were chosen for the study.

RESULTS

Immunoblot analysis

Affinity-purified antibodies directed against mGluR4a were first characterized by Western blotting analysis with membranes (25 μ g protein) from either wild type (WT) mouse brain or mouse brains from mutant mice lacking mGluR4 (Pekhletski et al., 1996). Figure 1 shows immunoblot analysis with polyclonal antibodies purified from two different rabbits. Consistent with our previous study (Bradley et al., 1996), both sets of antibodies reacted with a band of approximately 100 kDa in mouse and rat brain homogenates. This band directly corresponds with a band of the same molecular weight in cells expressing mGluR4a. In addition, both sets of antibodies react with a higher molecular weight band (approximately 200 kDa) that is

likely to represent a dimer of mGluR4a (Romano et al., 1996; Bradley et al., 1996).

Analysis of immunoreactivity in mutant mice lacking mGluR4 revealed that one set of antibodies is highly specific for mGluR4a and does not react with proteins in brains from the mGluR4 knockout (KO) mice (Fig. 1, left). However, other antibodies cross-react with an unknown protein in tissue from mGluR4a KO mice (Fig. 1, right). Interestingly, the cross-reactive product present in mGluR4 KO mice had the same molecular weight as mGluR4a in cell lines and wild-type mice, making detection of the cross-reactivity impossible without the availability of the mutant mice. These findings suggest that previous immunocytochemistry studies using the latter antibody preparation likely reflects reactivity with both mGluR4a and the unidentified protein (Bradley et al., 1996).

The more specific antibodies that did not react with proteins in mGluR4 KO mice were used for further characterization to determine whether these antibodies are specific for mGluR4a relative to other mGluR subtypes. Western blot analysis was performed with membranes from control (untransfected) cells, and cell lines (5 µg protein) transfected with either mGluR5, mGluR2, mGluR4a, mGluR4b, or mGluR7a. Affinity-purified antibodies directed against mGluR4a reacted with a band at about 100 KDa in homogenates of cells transfected with the mGluR4a but not with homogenates of cells transfected with other mGluR subtypes (Fig. 2). The mobility of this protein was consistent with the predicted molecular

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120 kDa

85

Fig. 2. Antibodies that specifically recognize metabotropic glutamate receptors (mGluR4a) in wild type mouse and nothing in mouse lacking mGluR4 do not cross-react with any other known mGluRs. Western blot analysis was used to determine the specificity of our antibodies for subtype 4a mGluRs. As shown in the figure, mGluR4a antibodies react with a band of about 100 kDa in homogenates from membranes of cell line transfected with mGluR4a, but mGluR4a immunoreactivity is absent in homogenates from cell lines transfected with mGluR2 (m2), mGluR5, mG), mGluR7a (m7a), mGluR4b (m4b), or untransfected cell line (Sf9). Molecular weight standards are designated on left in kilodaltons.



Fig. 3. Regional distribution of metabotropic glutamate receptor (mGluR4a) immunoreactivity in mouse brain. Western blot analysis was used to determine immunoreactivity with antibodies directed against mGluR4a in membranes (25 μ g of protein) from several regions of wild type mouse (+/+) brain. Regions analyzed included

neocortex (Ctx), cerebellum (Cereb), olfactory bulb (OB), piriform cortex/amygdala (Olf Ctx), pons/medulla (P/M), hippocampus (Hip), striatum (Striat), and midbrain (Midbr). These antibodies do not react with homogenates from membranes of mouse lacking mGluR4 (-/-).

weight of mGluR4a based on its amino acid sequence. Preadsorption of mGluR4a antibody with homologous peptide totally abolished all immunoreactive bands (not shown).

The regional distribution of mGluR4a immunoreactivity was determined in a number of dissected brain regions from WT and mGluR4 KO mice (Fig. 3). Antibodies directed against mGluR4a reacted with different intensities in several brain regions in WT mice. The strongest immunoreactivity was observed in the cerebellum. Immunoreactivity was also present in the cortex, striatum, olfactory cortex, and midbrain. Relatively weak immunoreactivity was detectable in the hippocampus, olfactory bub, and pons/medulla. Immunoreactive bands were not detected in any brain region from mGluR4a KO mice.

Immunocytochemical distribution of mGluR4a in mouse and rat brain

Affinity-purified antibodies that do not react with proteins in brains from the mGluR4 KO mice were used for immunocytochemical localization of mGluR4a in mouse and rat brain. Immunocytochemistry revealed specific staining with antibodies directed against mGluR4a in several brain regions in wild type mouse brain (Fig. 4A), whereas immunoreactivity was absent in brains from mice lacking mGluR4 (Fig. 4B). Immunoreactivity in WT mice was virtually abolished when the affinity-purified antibodies were preadsorbed with the homologous peptide (Fig. 4C). The same immunoreactive pattern was found in rat brain (not shown). Taken together with the high specificity of the antibodies demonstrated in the Western blot analysis, these data suggest that staining with these antibodies is highly selective for mGluR4a.

In the forebrain, mGluR4a showed a striking predilection for BG structures. Confocal fluorescence microscopy showed intense mGluR4a immunoreactivity in fibers in the GP (Fig. 4A). The STR overall exhibited very low intensity staining. Occasionally large labeled fibers were observed traversing the border of the STR with the GP (Fig. 4A). Only very low intensity was seen in the SNpr, and the large neurons of the substantia nigra pars compacta (SNpc) were not detectably labeled (Fig. 4A). In



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Fig. 5. Immunocytochemical staining of a section through the hippocampus and cerebellum in wild type and mutant mouse. Staining with antibodies directed against metabotropic glutamate receptor (mGluR4a) is shown in the hippocampus of wild type mouse (A) and mouse lacking mGluR4 (B). In wild type mouse, mGluR4a immunoreactivity was present in the middle and outer one-third of the molecular layer of the dentate gyrus (dg). Some staining was also present in the stratum oriens in CA3 area and in the stratum lacunosum-moleculare

these regions, mGluR4a immunoreactivity was highly localized on long processes, along which there was a intense staining of punta (Fig. 6). A moderate level of immunoreactivity was present in the STR.

the hippocampus proper. Axons in the fimbria (f) were also immunopositive for mGluR4a. As shown in B, immunoreactivity for mGluR4a is totally absent in mouse lacking mGluR4. In C is shown the staining for mGluR4a in the cerebellum. Very intense immunoreactivity product was present in the molecular layer (ml), whereas Purkinje cells (Pj) and granular layer (gl) were depleted of mGluR4a immunostaining. In mutant mouse cerebellum there was no immunoreactivity product (D). Scale bars = 0.4 mm in A, B; 0.1 mm in C, D.

Limbic cortical regions also showed relatively strong mGluR4a immunoreactivity. In the hippocampus (Figs. 4A, 5A), mGluR4a reaction product was visible in the outer and middle thirds of the molecular layer in the dentate gyrus. Some staining was also present in the neuropil of the stratum lacunosum-moleculare in area CA1 of the hippocampus proper and the stratum oriens in CA3 area. In the stratum pyramidale, mGluR4a immunoreactivity was noted in puncta surrounding the cell bodies of neurons and less frequently on cell bodies. This type of staining is also present in the granule cells of the dentate gyrus. Finally, mGluR4a immunoreactivity is present in the fimbria. The hippocampus of mice lacking mGluR4 was completly depleted of mGluR4a immunoreactivity (Fig. 5B). A moderate level of immunoreactivity was present in the piriform cortex (Pir), where immunoreactive fibers were present in layer 1A (Fig. 4A).

The highest level of mGluR4a immunoreactivity was found in the molecular layer of the cerebellum (Figs. 4A, 5C). In this region, intense staining was detected in fibers

Fig. 4. Immunocytochemical analysis of metabotropic glutamate receptor (mGluR4a) in mouse brain. A shows immunocytochemical analysis of staining with antibodies in sagittal sections of wild type mouse brain. This receptor was specifically present in only a few brain regions. The highest level of immunoreactivity was found in the molecular layer of the cerebellum. Intense staining was detected in fibers and puncta in the globus pallidus (GP) and substantia nigra pars reticulata (SNpr) and entopeduncular nucleus (ET). Some immunoreactivity product was also present in the hippocampus. Additionally, mGluR4a immunoreactivity was lower in regions such as the neccortex, thalamus, and striatum (STR). B shows that there was no staining in mouse lacking mGluR4. C shows that the staining was completely abolished when the mGluR4a antibodies were preadsorbed with 10 μ g/ml of homologous peptide. Pir, piriform cortex; Pr5VL, principal sensory trigeminal nucleus; Sp5, spinal trigeminal tract; VLL, ventral nucleus of the lateral lemniscus. Scale bar = 0.5 mm.

and neuropil of the molecular layer. In contrast, the cells of the granule layer were virtually devoid of mGluR4a immunoreactivity, as were the cells bodies and dendrites of the Purkinje cells. In the same area, mGluR4a immunoreactivity was absent in mice lacking mGluR4 (Figs. 4B, 5D). Moderate staining was also present in the ventrolateral part of the principal sensory trigeminal nucleus (Pr5VL; Fig. 4A).

Weak mGluR4a immunoreactivity was also present in the spinal trigeminal tract (Sp5) and in the ventral nucleus of the lateral lemniscus (VLL). mGluR4a immunoreactivity was low in several brain regions, including neocortex and thalamus (Fig. 4A).

mGluR4a immunoreactivity in the BG

To further address the pre-vs. postsynaptic distribution of mGluR4a immunoreactivity in rat BG, more detailed fluorescence and electron microscopic studies were performed. Confocal fluorescence microscopy showed intense mGluR4a immunoreactivity in fibers in the GP (Fig. 6). The STR overall exhibited very low intensity staining. Occasional large labeled fibers were observed traversing the border of the STR with the GP. Only very low intensity staining was seen in the SNpr, and the large neurons of the SNpc were not detectably labeled. No mGluR4a immunoreactivity associated with the somata was observed in any of these BG nuclei.

Double label experiments with MAP2 (Huber and Matus, 1984), a dendritic marker (Fig. 7A, B: mGluR4a staining in red in each of the panels of Fig. 7) showed a selective concentration of mGluR4a staining along the margin of certain dendrites in GP. At higher magnification (Fig. 7B), it was clear that most of the mGluR4a immunoreactivity was found on the dendritic surface, as it did not colocalize with MAP2 present within the dendrites. Figure 7C shows double label staining for mGluR4a and Enk. This study demonstrates that only a small subset of the Enk terminals label for mGluR4a. Figure 7D is a high magnification illustration showing double labeling with mGluR4a antibodies and the presynaptic marker SV2. mGluR4a and SV2 immunoreactivity are clearly colocalized along the outside of dendrites in the GP, suggesting a presynaptic localization of mGluR4a in GP. Consistent with this, Figures 7E and 7F show mGluR4a staining in the GP, contralateral (control) and ipsilateral to a quinolinate lesion of the striatum. Quinolinate lesioning of the projecting neurons to GP from the STR induced a marked decrease in mGluR4a immunoreactivity in ipsilateral (Fig. 7F) but not contralateral (Fig. 7E) GP. The residual staining for mGluR4a in the GP after striatal lesioning differed in appearance from the unlesioned side and that found in normal animals. It no longer outlined dendrites, but was instead found in coarse granules within the neuropil of the GP (Fig. 7E). These may represent staining associated with degenerating nerve terminals.

These double-label and lesion studies suggest that mGluR4a may be presynaptically localized on striatopallidal terminals. To further test this hypothesis, we performed immunocytochemistry with analysis at the electron microscopy level to clarify the pre- vs. postsynaptic localization of mGluR4a in the GP. Electron microscopy (EM) revealed that mGluR4a immunoreactivity is presynaptically localized (Fig. 8). Analysis of mGluR4a immunoreactivity at the EM level shows that the most intense staining was on axon terminals that form symmetric

synapses with dendrites (Fig. 8A-E). Although the majority of dendrites were not labeled, occasionally light staining of dendritic elements was observed (not shown).

DISCUSSION

In the present studies, we have characterized antibodies that are highly specific for mGluR4a and used them for immunocytochemical analysis of mGluR4a immunoreactivity in mouse and rat brain. Several experiments suggest that the antibodies react in a highly specific manner with the targeted receptor. Western blot analysis revealed that the antibodies selectively recognize proteins in cell lines transfected with mGluR4a but not in nontransfected cells or cell lines transfected with other mGluR subtypes. The antibodies also recognize a native protein in mouse and rat brain homogenates with a molecular weight consistent with the molecular weight of the immunoreactive protein in transfected cell lines. Furthermore, preadsorption of the antibodies with the homologous peptide abolished all the immunoreactive bands and immunocytochemical staining, suggesting that the antibodies react with the targeted epitope rather than binding nonspecifically. Finally, there was no immunoreactivity detected in brains of mutant mice lacking mGluR4. Taken together, these data suggest that these antibodies are highly specific for mGluR4a relative to other mGluR subtypes or other brain proteins.

In this paper, we focus on the localization of mGluR4a in BG because our immunocytochemical data and lesion studies reveal that mGluR4a has an important function as a presynaptic modulator in striatopallidal projections in the BG. The immunocytochemical analysis shows a striking immunoreactivity for mGluR4a in the GP, whereas SNpr and ET, the major output nuclei of the BG, were only weakly immunoreactive to mGluR4a. Finally, there was very low mGluR4a staining in STR or other BG structures. GP, SNpr, and ET each receive inhibitory GABAergic projections from the striatum (Fig. 9). Thus, the present data, coupled with previous in situ hybridization studies (Testa et al., 1994), suggest that mGluR4a could be synthesized in striatal neurons and targeted to presynaptic terminals of striatal projections to these other BG structures. Dual-label in situ studies on mGluR4 mRNA in striatal neurons reveal that the mRNA is present in all striatal projection neurons (Kerner et al., 1997). However, there is little mGluR4a immunoreactivity in the STR. Furthermore, mGluR4a seems to be found only on some of the striatopallidal terminals. These two observations suggest that either only some striatal neurons synthesize the protein, or all of them synthesize mGluR4a, but target it to only selected synapses with a subset of pallidal dendrites. Consistent with this latter hypothesis, in double labeling experiments with MAP2, mGluR4a staining was found clustered along a subset of the dendritic structures. All of the fibers displaying mGluR4a staining also exhibited colocalized staining for Enk, but there were many Enkpositive pallidal fibers and terminals which did not stain for mGluR4a. Thus, it is likely that mGluR4a is present in only a subpopulation of striatopallidal terminals. Double labeling immunocytochemistry revealed colocalization of mGluR4a immunoreactivity with the presynaptic marker synaptophysin. Furthermore, quinolinic acid lesions of the striatum virtually abolished mGluR4a immunoreactivity in GP. Finally, immuno-EM studies revealed that mGluR4a in GP is predominantly localized in presynaptic terminals



Fig. 6. mGluR4a immunoreactivity in basal ganglia. A shows intense localization of metabotropic glutamate receptor (mGluR4a) in fibers in the rat globus pallidus (GP). In contrast, the striatum (STR) is practically devoid of mGluR4a immunoreactivity. **B** shows a light

mGluR4a immunoreactivity in the substantia nigra part reticulata (SNpr), and a very light immunoreactivity in the substantia nigra pars compacta (SNpc). Scale bar = 50 mm.



Fig. 7. Double and single labeling confocal microscopy in globus pallidus (GP). A shows the selective concentration of metabotropic glutamate receptor (mGluR4a) staining (red) along the outside of certain dendrites (green), labeled with the postsynaptic marker micro-tubule-associated protein 2 (MAP2). B shows at higher magnification the colocalization of mGluR4a and MAP2. C shows staining for enkephalin (Enk; green), demonstrating that only a few Enk terminal

terminals are labeled for mGluR4a (red). **D** is a high magnification photo showing the colocalization (arrows) outside a dendrite of the presynaptic marker synaptic vesicle protein 2 (SV2; green) and mGluR4a (red), suggesting the presynaptic localization of mGluR4a in GP. **E** and **F** are illustrations of mGluR4a staining (red) in GP, contralateral (E) and ipsilateral (F) to a quinolinate lesion of the striatum. Scale bars = 10 mm in A, C; 5 mm in B, D; 100 mm in E, F.

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Fig. 8. A-E: Electron micrographs demonstrating presynaptic metabotropic glutamate receptor (mGluR4a) immunoreactivity in the globus pallidus (GP). Examples of mGluR4a axon terminals (asterisks) synapsing with dendrites (d) of cells in the GP. Scale bar = 400 nm in A- D; 350 nm in E.



Fig. 9. Summary figure illustrating results of the present and previous studies of metabotropic glutamate receptor mGluR localization in basal ganglia. Illustrated in schematic form are the two principal sources of afferent projections to striatum (STR), the cerebral cortex, which employs glutamate (GLU) as a transmitter, and the dopaminergic (DA) projection from the substantia nigra pars compacta (SNpc). The striatal projection neurons employ g-amino-nbutyric acid (GABA) as a neurotransmitter, and project to the output nuclei entopeduncular nucleus/substantia nigra part reticulata (ET/ SNpr/ET) either directly, or through a disynaptic relay involving the

that resemble striatal boutons and form symmetric synapses with GP dendrites and spines. Taken together, these data provide strong evidence that mGluR4a is localized on striato-pallidal terminals synapsing onto a subset of pallidal dendrites.

A comparison between these results and recent studies from our laboratories of mGluR7a localization in BG (Kosinski et al., 1999) reveals a number of similarities between the distribution of mGluR4a and mGluR7a. For instance, double label in situ hybridization studies revealed that mGluR7 mRNA is abundantly expressed in striatal projection neurons but not in striatal interneurons. As in the present experiments with mGluR4a, double labeling immunocytochemistry revealed colocalization of mGluR7a immunoreactivity with the presynaptic marker synaptophysin, and quinolinic acid lesions of the striatum resulted in the loss of mGluR7a immunoreactivity in GP. Finally, immuno-EM studies revealed that mGluR7a immunoreactivity is present in presynaptic terminals at symmetric synapses in GP. However, there are also several important differences between mGluR4a and mGluR7a immunoreactivity. For instance, mGluR7a immunoreactivglobus pallidus (GP) and the subthalamic nucleus (STN). Subthalamic efferents provide excitatory glutamatergic input to the GP and SNpr. In this schematic, the labels 1 to 7 refer to mRNAs encoding the mGluRs, whereas the symbols (+, ++, and +++) indicate the relative intensity of expression as determined by hybridization. Immunoreactivity is represented as *mGluR-mGluR7*. mGluR1 (most likely the mGluR1d isoform) is found on the dendrites of dopamine neurons in the SNpc, but appears not to be present on the axonal processes on these cells. Intense mGluR4a and mGluR7a immunoreactivity was detected in association with striatal terminals in GP.

ity is intense in STR, and lesion studies suggest that this represents staining at corticostriatal synapses. In contrast, there is little mGluR4a immunoreactivity in STR. Also, mGluR4a immunoreactivity is abundant in GP (likely at striatopallidal synapses) but relatively weak in substantia nigra (SN). In contrast, mGluR7a immunoreactivity is equally strong in SN and GP. Also, EM analysis revealed both pre- and postsynaptic labeling for mGluR7a in both GP and STR, whereas mGluR4a immunoreactivity was almost exclusively presynaptic.

Taken together, the present studies and the previous studies suggest that both mGluR4a and mGluR7a are localized in presynaptic terminals of striatopallidal projections. Previous studies show that a common function of mGluRs observed in other brain regions is a role as presynaptic heteroreceptors on inhibitory nerve terminals involved in inhibition of GABA release (Desai and Conn, 1991; Calabresi et al., 1992; Desai et al., 1994; Stefani et al., 1994). Thus, it is possible that presynaptically localized mGluR4a and mGluR7a could serve as heteroreceptors involved in regulating GABA release from striatopallidal and striato-SNpr terminals. The possibility that

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different mGluR subtypes may serve as heteroreceptors at different synapses is particularly intriguing and raises the possibility of selectively targeting the mGluR subtypes that serve as heteroreceptors in specific neuronal circuits relevant for various disorders. For instance, as a potential treatment for PD, agonists of group III receptors would reduce GABAergic inhibition of GP neurons, leading to an increase in firing of GABAergic GP neurons that project to the STN. This increased GABAergic inhibition of STN neurons would reduce STN neuronal firing and could normalize the over activity of STN neurons that occurs in PD. If so, these mGluRs could provide novel targets for new therapeutic agents that could be useful in treatment of PD and other disorders of BG function. However, the therapeutic benefit of group III mGluR agonists may ultimately depend on the balance of effects in GP and SNpr. If group III mGluRs also serve as presynaptic heteroreceptors on GABAergic terminals in SNpr, this could counteract the beneficial effect of activation of these receptors in GP.

Although the antibodies used for immunocytochemical analysis in the present study were highly specific for mGluR4a, other antibodies raised against the same epitope showed clear cross-reactivity with a band in homogenates from mutant mice that lacked mGluR4a. Interestingly, this cross-reactive band comigrated with mGluR4a, making it impossible to distinguish from mGluR4a except in the mutant mice. In a previous study (Bradley et al., 1996), we reported a regional and cellular distribution of immunoreactivity with mGluR4a-directed antibodies that appear to cross-react with this band in mGluR4 mutant mice. Western blot analysis with the previously characterized antibodies was relatively uniform in most brain regions and was clearly different from the distribution of mGluR4 mRNA (Tanabe et al., 1993). Immunocytochemical staining with these antibodies was also distinct from that reported here, and mGluR4a appears to have a more restricted distribution to presynaptic sites then we previously reported.

The identity of the mGluR4a cross-reactive protein is unknown. However, there are several interesting properties of the protein that raise the possibility that this protein it might be related to the mGluRs. First, it shares immunological features with mGluR4a. Second, the crossreaction protein has essentially identical mobility of mGluR4a by SDS-PAGE, indicating that their molecular weights are very similar. Third, the appearance of both bands is typical of glycosylated proteins. Fourth, ultrastructural analysis of the distribution of the cross-reacting protein implies that it is a membrane protein and postsynaptically localized at putative glutamatergic synapses. Taken together, these data raise the possibility that this protein may be related to mGluRs and may serve a postsynaptic function at glutamatergic synapses. Because the antibody does not cross-react with any known mGluRs in transfected cells, it may represent a novel mGluR or mGluR-like protein.

In summary, the present data suggest that mGluR4a has an abundant, but highly localized, distribution in BG. Taken together with previous studies focused on other mGluR subtypes, these data suggest that mGluRs are richly distributed in BG structures where they could play important roles in regulating the net output of these structures. Figure 9 summarizes our current understanding of mGluR localization in BG. Group III mGluRs (mGluR4 and mGluR7) are predominantly localized on presynaptic terminals of striatal projections to GP and SNpr (present studies; Kosinski et al., 1999). Group II mGluRs are presynaptically localized on corticostriatal projections and are expressed in STR (mGluR3) and STN (mGluR2), suggesting that they could play a role in these regions or in terminals of neurons originating from these areas, such as GP and SNpr (Testa et al., 1998). Finally, group I mGluRs are abundantly expressed in many of the BG nuclei (SNpc, SNpr, STR, and GP) where they are likely to serve as postsynaptic receptors involved in regulating neuronal excitability (Testa et al., 1998). If future electrophysiology studies verify the physiological roles of these receptors that would be predicted from these anatomical studies, this will provide a strong basis for development of novel therapeutic agents that target specific mGluR subtypes and could be used for treatment of PD and other disorders involving pathological changes in BG function.

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APPENDIX XI

NEURO

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Activation of groups I or III metabotropic glutamate receptors inhibits excitatory transmission in the rat subthalamic nucleus

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Abstract

The subthalamic nucleus (STN) is a key nucleus in the basal ganglia motor circuit that provides the major glutamatergic excitatory input to the basal ganglia output nuclei. The STN plays an important role in the normal motor function, as well as in pathological conditions such as Parkinson's disease. Development of a complete understanding of the role of the STN in motor control will require a detailed understanding of the mechanisms involved in the regulation of excitatory and inhibitory synaptic transmission in this nucleus. Here, we report that activation of groups I or III metabotropic glutamate (mGlu) receptors, but not group II, causes a depression of excitatory transmission in the STN. In contrast, mGlu receptor activation has no effect on the inhibitory transmission in this nucleus. Further characterization of the group I mGlu receptor-induced effect on EPSCs suggests that this response is mediated by mGlu1 and not mGlu5. Further, paired pulse studies suggest that both the mGlu1 receptor and the group III mGlu receptor-mediated effects are due to a presynaptic mechanism. If these receptors are involved in endogenous synaptic transmission in the STN, these results raise the exciting possibility that selective agents targeting mGlu receptors may provide a novel approach for the treatment of motor disorders involving the STN. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Metabotropic glutamate receptor; mGlu; Subthalamic nucleus; Basal ganglia; Parkinson's disease; Synaptic transmission

1. Introduction

The subthalamic nucleus (STN) is a critical region of the basal ganglia that is involved in the regulation of movement. The STN is the only excitatory glutamatergic nucleus in the basal ganglia motor circuit and provides the major excitatory input to the output nuclei, the substantia nigra pars reticulata and globus pallidus internal capsule. Normal motor function requires an intricate balance between excitation of the output nuclei by glutamatergic neurons from the STN, and inhibition of the output nuclei by GABAergic projections from the striatum (for a review see Wichmann and DeLong, 1997). Because of this, a great deal of effort has been focused on

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developing a detailed understanding of the circuitry and function of the STN.

Interestingly, recent studies suggest that the major pathophysiological change that occurs in response to loss of nigrostriatal dopamine neurons in Parkinson's disease patients is an increase in activity of STN neurons (Wichmann and DeLong, 1997). The resultant increase in synaptic excitation of GABAergic projection neurons in the output nuclei leads to a 'shutdown' of thalamocortical projections and produces the motor impairment characteristic of Parkinson's disease (DeLong, 1990). Discovery of the pivotal role of increased STN activity in Parkinson's disease has led to a major interest in the development of novel treatment strategies by reducing the neuronal STN activity or STN-induced excitation of basal ganglia output nuclei. Interestingly, surgical lesions (Bergman et al., 1990; Aziz et al., 1991; Guirdi et al., 1996), or inactivation of the STN (Benazzouz et al., 1993; Limousin et al., 1995a,b) are highly effective in the treatment of Parkinson's disease. Development of

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a detailed understanding of the mechanisms involved in the regulation of STN activity could lead to the development of novel therapeutic agents that reduce STN activity without surgical intervention.

Recent studies suggest that metabotropic glutamate (mGlu) receptors play an important role in regulating the activity of neurons in a wide variety of brain regions (for review see Anwyl, 1999; Cartmell and Schoepp, 2000). This has also been demonstrated extensively in basal ganglia structures such as the striatum (Calabresi et al., 1993; Colwell and Levine, 1994; Pisani et al., 1997b), the substantia nigra reticulata (Marino et al., 1999), the substantia nigra pars compacta (Fiorillo and Williams, 1998), the globus pallidus external segment (Maltseva and Conn, 2000), and the STN (Abbott et al., 1997; Awad et al., 2000). If mGlu receptors are involved in regulating synaptic transmission in the STN, this could provide a critical component of regulation of STN activity by glutamatergic afferents from the cortex, pedunculopontine nucleus, and thalamus, or GABAergic afferents from the globus pallidus external segment (Féger et al., 1997). Thus, it will be important to determine whether mGlu receptor activation modulates synaptic transmission in the STN. To date, eight mGlu receptor subtypes have been cloned from mammalian brain and are classified into three major groups based on sequence homologies, second messenger coupling and pharmacological profiles (see Conn and Pin, 1997 for review). Group I mGlu receptors (mGlu1 and mGlu5) couple primarily to Gq, whereas group II (mGlu2 and mGlu3) and group III mGlu receptors (mGlu 4, 6, 7, and 8) couple to Gi/Go. We now report that activation of groups I or III mGlu receptors leads to reduction of excitatory transmission in the STN.

2. Materials and methods

2.1. Materials

L(+)-2-Amino-4-phosphonobutyric acid (L-AP4), (S)- $(+)-\alpha$ -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (R, S)- α -cyclopropyl-4-phosphophenylglycine (CPPG), D(-)-2-amino-5-phosphonopentanoic acid (D-AP-5), 3,5-dihydroxyphenylglycine (DHPG), and methylphenylethynylpyridine (MPEP) were obtained from Tocris Cookson (Ballwin, MO). (+)-2-Aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740) was a gift from D. Schoepp (Eli Lilly, Indianapolis, IN). All other materials were obtained from Sigma (St. Louis, MO).

2.2. Tissue preparation

Experiments were performed on STN neurons from 15 to 18 day-old Sprague-Dawley rats. Rats were anes-

thetized with 7 mg/kg intraperitoneal injection of chloral hydrate prior to decapitation. The brain was removed, mounted and immersed in an oxygenated sucrose–ACSF solution containing: 3 mM KCl, 1.9 mM MgSO₄, 1.2 mM KH₂PO₄, 2 mM CaCl₂, 187 mM sucrose, 20 mM glucose, 26 mM NaHCO₃,0.5 mM pyruvate, 0.005 mM glutathione, equilibrated with 95% O₂ and 5% CO₂ at pH 7.4. Parasaggital slices of 300 μ m thickness are prepared using a manual VibroSlice (Stoelting, Chicago, IL) and then incubated at room temperature in ACSF containing): 124 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 1 mM NaH₂PO₄, 2 mM CaCl₂, 20 mM glucose, 26 mM NaHCO₃, 0.5 mM pyruvate, 0.005 mM glutathione, equilibrated with 95% O₂ and 5% CO₂ at pH 7.4.

2.3. Electrophysiological recordings

After a 2 h incubation at room temperature, the slices were transferred to a recording chamber mounted on the stage of a Hoffman modulation contrast Olympus microscope and continuously perfused with room temperature oxygenated ACSF (at 1-2 ml/min). Neurons in the STN were visualized using a water-immersion 40× objective. Whole cell patch clamp recordings were made using patch electrodes pulled from borosilicate glass on a Narishige vertical puller. Electrodes were filled with: 140 mM potassium gluconate, 10 mM HEPES, 10 mM NaCl, 0.2 mM EGTA, 2 mM MgATP, 0.2 mM NaGTP. Electrode resistance was $3-5 \text{ M}\Omega$. For measurement of synaptically evoked currents, bipolar tungsten electrodes were used to apply stimuli to the internal capsule rostral to the STN at 0.4-12.0 µA every 30-60 s. Synaptically evoked currents were recorded from a holding potential of -60 mV. Slices were bathed in 10 µM bicuculline to block inhibitory transmission for recording EPSCs, and 10 µM D-AP-5 and 20 µM CNQX to block excitatory transmission for recording IPSCs. For paired-pulse facilitation studies, stimuli were given in pairs with intervals ranging from 30 to 50 ms. All drugs were bath applied for 3-5 min in ACSF at 1-2 ml/min. Signals were recorded using a Warner PC-501A patch clamp amplifier (Warner Instrument Corporation, Hamden, CT) and pClamp data acquisition and analysis system (Axon Instruments, Foster City, CA).

2.4. Data analysis

Concentration-response data were fitted with a sigmoidal function using a Marquardt-Levenberg algorithm as implemented in the SigmaPlot program software (SPSS Inc., Chicago, IL). All values are expressed as mean±SEM. Statistical significance was assessed using the Mann-Whitney Rank Sum test.

3. Results

3.1. Groups I or III mGlu receptor activation reduces EPSC amplitude in the STN

A single stimulus to the internal capsule, rostral to the STN induced an inward synaptic current in the presence of 10 μ M bicuculline at a holding potential of -60 mV. EPSCs were completely blocked by 20 µM CNQX suggesting that the EPSC is primarily mediated by nonNMDA receptors (data not shown). The group I mGlu receptor-selective agonist, DHPG (100 µM) (Schoepp et al., 1994) caused a transient, reversible depression of EPSCs in STN neurons (Fig. 1A). The group II selective agonist LY354740 (100 nM) (Monn et al., 1997) had no effect on the EPSC amplitude in the STN (Fig. 1B). The group III-selective agonist L-AP4 (1 mM) (Pin and Duvoisin, 1995) also caused a reversible depression of EPSCs in the STN (Fig. 1C). Time courses for each of the group-selective agonist effects on EPSCs are shown (Fig. 1D-F). The DHPG effect is transient and begins desensitizing in the continued presence of the agonist (Fig. 1D). The L-AP4-induced EPSC inhibition showed no apparent desensitization over the period of drug application. However, within approximately 15–20 min of washout of L-AP4, the EPSC returns to within ~80% of predrug amplitude (Fig. 1F).

Concentration-response analysis revealed that both the DHPG and L-AP4 induced inhibition of EPSCs in the STN were concentration-dependent (Fig. 2A and B). The maximal DHPG inhibition was $46.6\pm3.2\%$ and occurred at 50 μ M (Fig. 2A). L-AP4 is more effective at inhibiting EPSCs with a maximal EPSC inhibition of $85.6\pm1.3\%$ at a concentration of 500 μ M (Fig. 2B).

3.2. mGlu receptor activation has no effect on the inhibitory transmission in the STN

Internal capsule stimulation in the presence of 20 μ M CNQX and 10 μ M D-AP-5 evoked an outward IPSC that was blocked by 10 μ M bicuculline (data not shown). DHPG (100 μ M) (92.3 \pm 23.6% control), LY354740 (100 nM) (98.4 \pm 8.8% control) and L-AP4 (1 mM) (99.8 \pm 17.6% control) were all without effect on IPSC amplitude in the STN (Fig. 3A–C). Time courses for each of the drug applications are shown indicating no



Fig. 1. Activation of groups I or III mGluRs reduces EPSCs in the STN. (A–C) Representative voltage clamp traces of evoked EPSCs in the STN before, during, and after a 3-min application of DHPG (100μ M), LY354740 (100 nM), or L-AP4 (1 mM). (D–F) Average time-course of the effect of each agonist on the normalized EPSC amplitude (agonist application is indicated by a horizontal bar). Each time point represents the mean (±SEM) of data from seven cells for DHPG, five cells for LY354740, and four cells for L-AP4.



Fig. 2. Concentration-response relationship of groups I and III induced inhibition of EPSCs in the STN. (A) Concentration-response relationship of DHPG-induced inhibition of EPSCs. Maximal DHPG response occurs at a concentration of $50 \,\mu$ M. Each point represents the mean (±SEM) of 3–7 experiments. (B) Concentration-response relationship of L-AP4-induced inhibition of EPSCs. Maximal L-AP4 response occurs at a concentration of $500 \,\mu$ M. Each point represents the mean (±SEM) of 3–4 experiments.

effect of any of the group-selective mGlu receptor agonists on IPSC amplitude (Fig. 3D–F).

3.3. mGlu receptor-induced inhibition of EPSCs in the STN is blocked by selective antagonists

We utilized the newly available pharmacological tools that distinguish between mGlu1 and mGlu5 receptor subtypes to characterize further the group I mediated inhibition of EPSCs in the STN. For these studies, antagonists were bath applied for approximately 10 min prior to the application of the agonist. The mGlu1-selective competitive antagonist LY367385 (300 μ M) (Clark et al., 1997; Bruno et al., 1999; Kingston et al., 1999) completely blocked the depression of EPSCs induced by DHPG (10 μ M) (3.4±4.88% inhibition, vs 25.2±3.96% inhibition, respectively) (Fig. 4A, B and D). On the other hand, MPEP (10μ M), a noncompetitive antagonist at mGlu5 (Bowes et al., 1999; Gasparini et al., 1999), had no effect on the DHPG-induced inhibition of EPSCs ($28.8\pm10.2\%$ inhibition) (Fig. 4A, C and D). This provides strong evidence that the group I mGlu receptor subtype mediating depression of EPSCs in the STN is mGlu1.

A 10-min preincubation of the group II/III mGlu receptor antagonist CPPG (500 μ M) (Toms et al., 1996) blocked the L-AP4-induced inhibition of EPSCs in the STN (31.0±13.8% inhibition, vs 85.6±1.3% inhibition, respectively) (Fig. 5). Since group II mGlu receptors were without effect on the EPSC amplitude in the STN, this provides sufficient evidence that this depression of EPSCs is in fact mediated by group III mGlu receptors.

3.4. The effect of groups I and III selective agonists on EPSCs is mediated by a presynaptic mechanism

To test the hypothesis that group I or III mGlu receptor activation induces a depression of the excitatory synaptic transmission in the STN by a presynaptic mechanism, we determined the effects of group-selective agonists on paired-pulse facilitation of evoked EPSCs. Paired stimulations of the internal capsule were performed at the same stimulus strength with intervals ranging from 30 to 50 ms. Stimulus strength and interpulse intervals were adjusted in each experiment so that the second EPSC was always greater in amplitude than the first (ratio of EPSC2/EPSC1: 1.44±0.117). DHPG (100 µM) reduced the absolute amplitude of the first EPSC, but also increased the ratio of paired-pulse facilitation (ratio of EPSC2/EPSC1: 2.47±0.246) (Fig. 6A and E) significantly. Similarly, L-AP4 (500 µM) also caused a reduction in the EPSC amplitude concomitant with an in paired-pulse facilitation (ratio of increase EPSC2/EPSC1: 3.36±0.463) (Fig. 6B and E). Representative time-courses of the paired pulse facilitation data (Fig. 6C-D) show that the increase in the paired pulse ratio occurs concomitantly with the EPSC inhibition time-courses shown in Fig. 1D and F. In addition, our previously published findings show that neither group I nor group III mGlu receptor activation have any effect on the postsynaptic kainate-evoked current amplitude (Awad et al., 2000). These data combined provide strong evidence in support of the hypothesis that groups I and III mGlu receptors act presynaptically to inhibit the evoked release of glutamate from excitatory glutamatergic terminals in the STN.

4. Discussion

The data presented in this study show that activation of group I or III mGlu receptors reduces the excitatory glutamatergic transmission in the STN, and that this



Fig. 3. Activation of groups I, II or III mGluRs has no effect on IPSCs in the STN. (A–C) Representative voltage clamp traces of evoked IPSCs in the STN before, during, and after a 5-min application of DHPG (100μ M), LY354740 (100 nM), or L-AP4 (1 mM). (D–F) Average time-course of the effect of each agonist on the normalized IPSC amplitude (agonist application is indicated by horizontal bar). Each time point represents the mean (±SEM) of data from four cells for DHPG, six cells for LY354740, and three cells for L-AP4.

reduction is likely mediated by a presynaptic mechanism. We also show that mGlu receptors do not play a role in modulating inhibitory transmission in the STN.

Our current findings add to a growing body of literature suggesting that group I mGlu receptors play an important role in regulating basal ganglia function. Group I mGlu receptor mRNA and protein were shown to be expressed throughout the basal ganglia (Testa et al. 1994, 1998; Kerner et al., 1997; Tallaksen-Greene et al., 1998). Both subtypes of group I mGlu receptors are localized postsynaptically in the STN and group I mGlu receptor agonists induce a profound excitation of STN neurons (Abbott et al., 1997; Awad et al., 2000). Group I mGlu receptors, are also heavily localized in the striatum (Shigemoto et al., 1993; Tallaksen-Greene et al., 1998) and substantia nigra pars reticulata (SNr) (Hubert and Smith, 1999) where agonists of these receptors induce excitatory effects (Calabresi et al., 1992; Colwell and Levine, 1994; Pisani et al., 1997b; Marino et al., 1999). In the dopaminergic neurons of the substantia nigra pars compacta (SNc), group I mGlu receptor activation has been shown to elicit a transient hyperpolarization followed by a more pronounced depolarization (Fiorillo and Williams, 1998), and induce a depression of EPSPs (Wigmore and Lacey, 1998). Behavioral studies combined with studies of changes in 2-deoxyglucose and fos immunoreactivity suggest that injection of group I mGlu receptor agonists into the striatum or the STN induces rotational behavior (Sacaan et al. 1991, 1992; Kaatz and Albin, 1995; Feeley Kearney et al., 1997). Taken together, these data suggest that group I mGlu receptors function at multiple levels of the basal ganglia circuit to regulate activity of neurons in the STN and output nuclei.

While group I mGlu receptor agonists induce a number of physiological responses in the basal ganglia, recent studies with the newly developed mGlu1 receptor and mGlu5 receptor ligands suggest that the physiological roles of the group I mGlu receptor subtypes are highly segregated. In the SNr, both mGlu1 and mGlu5 receptor subtypes are localized postsynaptically (Hubert and Smith, 1999). However, mGlu1 mediates the physiological effects of group I mGlu receptor activation in this nucleus (Marino et al., 1999). On the other hand, the converse is true in the STN where both mGlu1 and mGlu5 receptor subtypes are postsynaptically localized, but all physiological effects of group I mGlu receptor activation are mediated by mGlu5 (Awad et al., 2000). Further, our studies indicate that group I mGlu receptor activation in the SNr causes an inhibition of IPSCs



Fig. 4. The group I-induced inhibition of EPSCs is mediated by mGluR1. (A) Representative voltage clamp EPSC traces before, during, and after application of DHPG (10 μ M). (B) Effect of a 10-min preapplication of the mGluR1-selective antagonist LY367385 (300 μ M) on the DHPG-induced inhibition of EPSCs. (C) Effect of a 10 minute preapplication of the mGluR5-selective antagonist MPEP (10 μ M) on the DHPG-induced inhibition of EPSCs. (D) Mean data±SEM of % EPSC inhibition by DHPG (10 μ M) in the absence and presence of selective antagonists. Each bar represents average data from four cells (*p<0.05, Mann–Whitney test).

(Wittmann et al., 2000) which is believed to be mediated by both mGlu1 and mGlu5 receptor subtypes (unpublished observations).

The present finding that mGlu1 regulates excitatory synaptic transmission in the STN is especially interesting in light of the fact that group I mGlu receptors have been traditionally viewed as primarily postsynaptic receptors that play roles in modulation of postsynaptic cell excitability. Increasing evidence suggests that group I mGlu receptors may also play important roles in the modulation of synaptic transmission at both excitatory and inhibitory synapses throughout the brain (Calabresi et al., 1992; Gereau and Conn, 1995; Bonci et al., 1997; Manzoni et al., 1997; Anwyl, 1999; Cartmell and Schoepp, 2000). Our finding that mGlu1 activation inhibits glutamate release in the STN is consistent with our previous finding of slight mGlu1a immunoreactivity on presynaptic terminals at asymmetric synapses in the STN (Awad et al., 2000). This reduction in excitatory transmission is interesting because it has the opposite effect on the activity of STN neurons than that seen postsynaptically by activation of mGlu5. This could provide a mechanism for differential modulation of STN neuron activity by selectively targeting either mGlu1 or mGlu5. It will be important to determine the net effect of group I mGlu receptor activation in light of these opposing effects in different basal ganglia regions.

Our finding that group III mGlu receptor activation leads to an inhibition of synaptic excitation in the STN is consistent with both the anatomical and physiological studies in other basal ganglia regions. Anatomical studies have demonstrated the presynaptic localization of the mGlu7 receptor subtype at corticostriatal terminals as well as striatopallidal terminals and striatonigral terminals (Kosinski et al., 1999). In addition, presynaptic localization of mGlu4 has been demonstrated at striatopallidal terminals (Bradley et al., 1999b). Group III mGlu receptors were shown to reduce excitatory transmission at the corticostriatal synapse (Pisani et al., 1997a) and at excitatory synapses in the SNc (Wigmore and Lacey, 1998). Group III mGlu receptors have also been shown to reduce both excitatory and inhibitory transmissions in the SNr (Wittmann et al., 2000). Unfortunately, drugs are not available to allow a clear determination of the specific group III mGlu receptor subtypes that mediate these effects. However, previous immunocytochemical studies reveal that mGlu4 and mGlu7 receptors are present in the STN (Bradley et al., 1999a,b; Kosinski et al., 1999), whereas mGlu6 and mGlu8 are not. Thus, the most likely candidates for group III mGlu receptors mediating this effect in the STN are mGlu4 and mGlu7.

Interestingly, group II mGlu receptor activation has no effect on the excitatory transmission in the STN. This is consistent with the previous reports indicating little or no mGlu2/3 immuno-reactivity in the STN (Testa et al., 1998). However, this is interesting in light of increasing evidence demonstrating the role of group II mGlu receptors in the modulation of transmission in several basal ganglia regions. Group II mGlu receptors are localized presynaptically at asymmetric synpases in the SNr and inhibit excitatory synaptic transmission at the STN–SNr synapse (Bradley et al., 2000). Group II mGlu receptors have also been shown to be expressed in the striatum and the SNc (Testa et al., 1998) and they reduce excitatory transmission in these nuclei (Lovinger and McCool, 1995; Wigmore and Lacey, 1998).

The major sources of glutamatergic afferents into the STN arise from the cortex, thalamus, and pedunculopontine nucleus (PPN) (Parent and Hazrati, 1995; Féger et al., 1997). Corticosubthalamic terminals were shown to H. Awad-Granko, P. Jeffrey Conn / Neuropharmacology 41 (2001) 32-41



Fig. 5. The group III-induced inhibition of EPSCs is attenuated by a groups II/III antagonist. (A) Representative voltage clamp EPSC traces before, during, and after application of L-AP4 (500 μ M). (B) Effect of a 10-min preapplication of the groups II/III mGluR antagonist CPPG (500 μ M) on the L-AP4-induced inhibition of EPSCs. (C) Mean data±SEM of % EPSC inhibition by L-AP4 (500 μ M) in the absence and presence of CPPG. Each bar represents average data from three cells (*p<0.05, Mann–Whitney test).



Fig. 6. Inhibition of EPSCs by activation of groups I and III mGluRs is mediated by a presynaptic mechanism. (A–B) Representative voltage clamp traces of paired evoked EPSCs before and during application of DHPG (100μ M) or L-AP4 (500μ M). Scaled overlay represents the superimposed traces of the predrug condition (solid line) and the agonist condition (dashed line; trace scaled to the first EPSC of control condition). (C–D) Representative time-courses of the paired pulse ratio data in one experiment each for DHPG (100μ M) and L-AP4 (500μ M). The dashed line indicates the average predrug ratio. (E) Mean data±SEM of the ratio of the second EPSC to the first in control (*n*=13), DHPG (*n*=8) and L-AP4 (*n*=5) conditions (***p<0.005, Mann–Whitney test).

be highly enriched with glutamate immunoreactivity (Bevan et al., 1995). Interestingly, the same postsynaptic structures in the STN that receive this cortical input also receive synaptic afferents from GABAergic pallidal-like terminals, suggesting that both glutamatergic and GABAergic afferents converge onto a single STN cell (Bevan et al., 1995). If this is the case, then it is likely that selective modulation of excitatory transmission in the STN with group I or III mGlu receptor agonists could lead to a reduction in the STN activity without affecting the inhibitory transmission. Electrical stimulation of either the PPN or the parafasicular thalamic nucleus (PF) have also been shown to induce an excitatory glutamatergic response in STN neurons (Hammond et al., 1983; Mouroux and Feger, 1993). Pharmacological stimulation of the PF with carbachol results in an increase in the discharge rate of STN neurons (Mouroux et al., 1995). However, in the present study it is important to note that we are unable to determine the source of the afferents being modulated by mGlu receptors. It is likely that by stimulating the internal capsule, we are stimulating afferents from multiple sources. Further studies are necessary to determine the sources of these afferents, and whether or not different afferents have differential mGlu receptor expression and show differential mGlu receptor actions.

Taken together, these studies demonstrate that groups I and III mGlu receptors are involved in the reduction of excitatory transmission in the STN. These receptors may provide exciting new targets for the development of pharmacological treatments of disorders caused by an alteration in the activity of the STN, such as Parkinson's disease, Huntington's disease, and Tourette's syndrome. By selectively targeting different mGlu receptor subtypes with specific mGlu receptor agonists or antagonists it may be possible to restore the balance necessary for normal basal ganglia function. However, it is important to note that the use of antagonists of mGlu receptors to treat these various disorders will depend upon the physiological activation of these receptors by endogenous glutamate, which is yet to be demonstrated. In addition, owing to the differential effects of mGlu receptor activation in the different regions of the basal ganglia, it will be important to determine the overall net effect of activation or inhibition of mGlu receptors in the whole animal prior to the consideration of agents modulating these receptors for therapeutic use.

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Haloperidol-induced alteration in the physiological actions of group I mGlus in the subthalamic nucleus and the substantia nigra pars reticulata

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Abstract

Excitatory glutamatergic inputs to the subthalamic nucleus (STN), and subthalamic afferents to the substantia nigra pars reticulata (SNr) are believed to play a key role in the pathophysiology of Parkinson's disease (PD). Previously, we have shown that activation of the group I mGlus in the STN and SNr induces a direct depolarization of the neurons in these nuclei. Surprisingly, although both group I mGlus were present in the STN and SNr, mGlu5 alone mediated the DHPG-induced depolarization of the STN, and mGlu1 alone mediated the DHPG-induced depolarization of the SNr. We now report that both mGlu1 and mGlu5 are coexpressed in the same cells in both of these brain regions, and that both receptors play a role in mediating the DHPG-induced increase in intracellular calcium. Furthermore, we demonstrate that the induction of an acute PD-like state using a 16 h haloperidol treatment produces an alteration in the coupling of the group I receptors, such that post-haloperidol, DHPG-induced depolarizations are mediated by both mGlu1 and mGlu5 in the STN and SNr. Therefore, the pharmacology of the group I mGlu-mediated depolarization depends on the state of the system, and alterations in receptor coupling may be evident in pathological states such as PD. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Basal ganglia; Metabotropic glutamate receptor; Substantia nigra pars reticulata; Subthalamic nucleus; Dopamine; Movement disorder

1. Introduction

The glutamatergic synapse between the subthalamic nucleus (STN) and the basal ganglia (BG) output nuclei is of particular importance in the pathophysiology of Parkinson's disease (PD). Hyperactivation of the STN, and resultant increased BG outflow is a consistent feature of both clinical PD and animal models of the Parkinsonian state (DeLong, 1990; Bergman et al., 1990). Furthermore, surgical interventions that ablate or normalize the excessive STN or BG outflow are extremely effective at ameliorating the symptoms of PD (Bergman et al., 1990; Aziz et al., 1991; Limousin et al., 1995a, 1995b; Wichmann and DeLong, 1997). A thorough understand-

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ing of the neuromodulatory regulation and plasticity of this circuit may provide a better understanding of the pathophysiology of PD, as well as reveal novel targets for the treatment of this debilitating disorder.

Recent studies suggest that metabotropic glutamate receptors (mGlus) play important roles in regulating neuronal activity and function in a number of BG structures such as the striatum (Calabresi et al., 1992; Colwell and Levine, 1994; Lovinger and McCool, 1995; Pisani et al., 1997), STN (Kaatz and Albin, 1995; Abbott et al., 1997; Awad et al., 2000), substantia nigra pars reticulata (SNr) (Bradley et al., 2000; Marino et al., 2001; Wittmann et al., 2001a, 2001b), substantia nigra pars compacta (Mercuri et al., 1993; Meltzer et al., 1997; Shen and Johnson, 1997; Fiorillo and Williams, 1998; Wigmore and Lacey, 1998), and the globus pallidus (Maltseva and Conn, 2000; Poisik and Conn, 2001). To date, eight mGlu subtypes have been cloned from mammalian brain and are classified into three major groups based on sequence homologies, second messenger coupling and

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¹ M.J. Marino and H. Awad-Granko contributed equally to this paper.
pharmacological profiles (for review, see Conn and Pin, 1997; Cartmell and Schoepp, 2000). Group I mGlus (mGlu1 and mGlu5) couple primarily to Gq, whereas Group II (mGlu2 and mGlu3) and group III mGlus (mGlus 4, 6, 7, and 8) couple to Gi/Go. Previous studies from our laboratory have shown that activation of group I mGlus, using the highly selective group I mGlu agonist DHPG, produces a direct excitation of neurons in both the STN and the SNr (Awad et al., 2000; Marino et al., 2001). Interestingly, while immunocytochemical studies have demonstrated the presence of both mGlul and mGlu5 protein in the STN and SNr (Awad et al., 2000; Hubert et al., 2001), pharmacological investigations employing highly selective antagonists for mGlu1 and mGlu5 have uncovered distinct functions of these receptors (Awad et al., 2000; Marino et al., 2001). In STN neurons, the group I mGlu-induced depolarization is solely mediated by mGlu5 (Awad et al., 2000), while in SNr neurons, the DHPG-induced depolarization is solely mediated by mGlu1 (Marino et al., 2001). These findings were surprising since the coupling of these receptors in recombinant systems is identical, and colocalization of the two receptors is usually assumed to indicate a redundancy of function. In the present study, we employ immunocytochemistry and calcium imaging to demonstrate that both mGlu1 and mGlu5 colocalize within individual neurons in the STN and the SNr, and both receptors are involved in inducing calcium release in response to DHPG.

The distinct pharmacology of the mGlu-mediated depolarization in these two regions is likely to be due to a difference in their coupling to effector mechanisms. Group I mGlu coupling to effector molecules is under tight regulation by a number of regulatory proteins including protein kinase C (Schoepp and Johnson, 1988; Herrero et al., 1994; Gereau and Heinemann, 1998; Macek et al., 1998; Francesconi and Duvoisin, 2000), G-protein coupled receptor kinases (Dale et al., 2000; Sallese et al., 2000), regulators of G-protein signaling (Saugstad et al., 1998; Kammermeier and Ikeda, 1999), and various scaffolding/trafficking proteins such as Homer, Shank, and PSD-95 (Sheng, 1996; Brakeman et al., 1997; Kornau et al., 1997; Ziff, 1997; Craven and Bredt, 1998; Tu et al., 1998; Ciruela et al., 1999). It is possible that the differential coupling of mGlus in the STN and SNr could be under the dynamic control of any number of these regulatory proteins, and could subsequently be altered by the functional state of the system. Since PD is known to involve a dramatic increase in excitatory drive through the STN and SNr, we employed an overnight haloperidol treatment to mimic this increase in activity in order to determine whether the coupling of the group I mGlus in these two brain regions is altered in this pathological state. Our current findings indicate that 16 h haloperidol treatment alters the pharmacology of the group I-mediated depolarization in both the STN

and SNr by inducing both mGlu1 and mGlu5 to contribute to the depolarization in both nuclei. These findings indicate that a degree of plasticity exists in the coupling of group I mGlus and suggests that these receptors may play enhanced roles in mediating excitatory transmission under pathological conditions. These findings may shed some light on the functions of expression of multiple subtypes of closely related receptors by a single neuronal population.

2. Material and Methods

2.1. Immunocytochemistry

Sprague–Dawley rat pups (15–18 days) were used for all immunocytochemical studies. Animals were deeply anesthetized with isoflurane and perfused using a gravity perfusion apparatus with normal saline mixed with 0.005% sodium nitroprusside at room temperature for 1 min followed by ice-cold 4% *p*-formaldehyde in 0.1 M sodium phosphate, pH 7.4 for 10 min. The brains were immediately removed and postfixed in the same fixative overnight at 4°C. The pia matter was carefully removed and 50 μ m sections were cut in cold PBS using an OTS-4000 Tissue Slicer (Frederick Haer and Co., Bowdoinham, ME). Sections were then processed immediately for immunohistochemistry or stored in a solution of 30% sucrose, 0.1 M sodium phosphate and 30% ethylene glycol at -20°.

Sections were washed in phosphate buffered saline (PBS), incubated in 3% hydrogen peroxide/PBS (pH 7.4) for 10 min and blocked with avidin (10 µg/ml in PBS with 5% normal goat and 5% normal horse serum) for 30 min. The sections were washed in PBS and co-incubated overnight with purified mouse monoclonal IgG1 raised against the entire C-terminus of human mGlula (PharMingen, San Diego, CA), and purified rabbit polyclonal IgG1 raised against the mGlu5 C-terminus (KSSPKYDTLIIRDYTNSSSSL, Upstate Biotechnologies, Lake Placid, NY). Previous studies with these antibodies have demonstrated specificity and lack of crossreactivity both by immunoblotting and immunocytochemistry (Hubert et al., 2001; Marino et al., 2001). Antibodies were diluted to their final concentration (mGlula 1:2000, mGlu5 1:1000) using avidin (50 µg/ml with 1% normal goat serum and 1% normal horse serum) in PBS. Sections were then washed in PBS prior to addition of the fluorescent secondary antibodies. Antibodies to the mGlu5 receptors were labeled by using donkey anti-rabbit (1:100, Jackson Labs) coupled to rhodamine for 1 h, followed by additional washing and then incubated in anti-mouse (1:100,Jackson biotinylated goat Laboratories) for labeling of mGlula for another hour. Both secondaries were diluted in PBS with 1% normal goat serum and 1% normal horse serum. Sections were again washed and then incubated in ABC (1:500, Vector Elite) for 30 min prior to amplification with tyramide-FITC (1:100, Perkin-Elmer) for 10 min. The sections were then incubated for 30 min in 10 mM cupric sulfate and 50 mM ammonium acetate (pH 5.0) in water. All incubations were performed at room temperature. Sections were wet mounted on slides (Fisherbrand Superfrost/Plus) and coverslipped using Vector Vectashield mounting medium and stored in the dark at 4°C.

2.2. Slice preparation

Fifteen- to 18-day-old Sprague-Dawley rats were used for all patch clamp studies. Midbrain slices were prepared as previously described (Awad et al., 2000; Marino et al., 2001). After decapitation, brains were rapidly removed and submerged in an ice cold sucrose buffer (in mM): Sucrose, 187; KCl, 3; MgSO₄, 1.9; KH₂PO₄, 1.2; Glucose, 20; NaHCO₃, 26; equilibrated with 95% $O_2/5\%$ CO₂. Parasaggital slices (300 µm thick) were made using a Vibraslicer (WPI) or Vibratome (TPI). Slices were transferred to a holding chamber containing normal ACSF (in mM): NaCl, 124; KCl, 2.5; MgSO₄, 1.3; NaH₂PO₄, 1.0; CaCl₂, 2.0, Glucose, 20; NaHCO₃, 26; equilibrated with 95% O₂/5% CO₂. In all experiments, 5 µM glutathione and 500 µM pyruvate were included in the sucrose buffer and holding chamber to increase slice viability. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with ACSF (~3 ml/min, 32°C).

2.3. Electrophysiology

Neurons in the STN or SNr were visualized with a 40× water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with (in mM): potassium gluconate, 140; HEPES, 10; NaCl, 10; EGTA, 0.6; NaGTP, 0.2; MgATP, 2; pH adjusted to 7.4 with 0.5 N KOH. Electrode resistance was $3-7 \text{ M}\Omega$. For approximately half of the recordings from SNr, the intracellular solution was composed of (in mM): KCH₃SO₄ (115), NaCl (20), MgCl₂ (1.5), HEPES (5), EGTA (0.1), MgATP (2), NaGTP (0.5), phosphocreatine (10), and the pH adjusted to 7.5 with 0.5 N KOH. There was no detectable difference in these two groups, so the results were combined for analysis. GABAergic SNr neurons were identified according to previously established electrophysiological criteria (Richards et al., 1997). GABAergic neurons exhibited spontaneous repetitive firing, short duration action potentials, little spike frequency adaptation, and a lack of inward rectification, while dopaminergic neurons displayed no, or low frequency spontaneous firing, longer duration action potentials, strong spike frequency adaptation, and a pronounced inward rectification.

2.4. Fluo-3 bulk loading method

For calcium imaging in the STN, we employed a bulk loading method similar to that described previously by Singha and Saggau (1999). After preparation of slices for electrophysiology, slices were allowed to equilibrate for ca 1 h. The membrane permeable acetoxymethyl ester (AM) of Fluo-3 (50 µg, Molecular Probes, Eugene, OR) was dissolved in 10 µl of a solution consisting of 20% by weight pluronic acid in DMSO (Molecular Probes, Eugene, OR). This solution was allowed to sit for 30-60 min prior to use. Immediately prior to loading the slices, 200 µl of ACSF was added to the dissolved Fluo-3-AM and mixed rapidly. An additional 800 µl of ACSF was added, resulting in a final concentration of Fluo-3-AM of 40 µM. This solution was then transferred to a modified 24-well culture plate and the slices were added (2-3 slices/500 µl). A 25-gauge needle was inserted through the lid of the culture plate and positioned at an angle so that its tip was maintained just above the level of the fluid in the well. A 95% $O_2/$, 5% CO₂ mixture was gently passed through the needle and across the surface of the solution. The plate was then covered, sealed with parafilm, and placed in a 30°C water bath for 1.5 h. The slices were then transferred to normal ACSF and maintained in a standard holding chamber until use.

2.5. Fluo-3, whole-cell loading method

Since the SNr represents a heterogeneous population of neurons, we were not able to employ the bulk loading method. We therefore filled the cell with calcium indicator by diffusion from the whole cell patch pipette after confirming the GABAergic phenotype as described above. The intracellular solution for all calcium imaging experiments was composed of (in mM): K-methylsulfate (115), NaCl (20), MgCl₂ (1.5), CaCl₂ (0.2), HEPES (5), EGTA (0.4), MgATP (2), NaGTP (0.5), phosphocreatine (10), pH adjusted to 7.5 with 0.5 N KOH. 100 µM Fluo-3 pentapotassium salt (Molecular Probes) was dissolved in this intracellular solution just prior to use. After obtaining a whole cell recording, the indicator was allowed to diffuse into the cell for at least 20 min before beginning an experiment. Cells were maintained in voltage clamp mode at a holding potential of -60 mV, and series resistance was monitored throughout the duration of the experiment.

2.6. Calcium imaging

Calcium imaging was performed using a Till Photonics imaging system (Martinsried, Germany) obtained from ASI inc, (Eugene, OR) attached to an Olympus BX50WI inverted microscope. Cells were excited at 488 nM using the Polychrome IV monochrometer and fluorescence was recorded through a 500–550 nm band pass filter on a IMAGO CCD Camera. Images were acquired, stored and analyzed using the IMPULS TILLVISION v. 3.3 software (TILL Photonics, Martinsried, Germany). All imaging was done at 1 Hz using a 10–40 ms excitation pulse. In order to prevent any bias in the analysis of imaging data from bulk loaded slices, a random area of the STN measuring 100×100 μ m was pre-selected prior to the experiment and all subsequent measurements and analysis were performed on cells located within this area of interest (AOI). All calcium recordings were performed in the presence of 0.5 μ M tetrodotoxin to block action potential firing. Agonists were bath applied for 1– 2 min. Antagonists were bath applied for a period of 10 min prior to agonist application.

2.7. Haloperidol treatment

Osmotic pumps with a 200 µl capacity, calibrated to deliver 1 µl/h (Model No. 2001, Alzet, Durect Corporation, Cupertino, CA) were filled with a concentration of haloperidol sufficient to deliver a supramaximal dose of 6 mg/kg/day. Immediately before use, haloperidol (10 mg/ml) was dissolved in a solution of 6 parts 8.5% lactic acid, and then neutralized with 4 parts 1N NaOH. This solution was then diluted to final concentration in sterile saline. Rat pups (15-18 days old, 20-37 g) were lightly anesthetized with isoflurane and the pump was inserted subcutaneously through an incision made ~1 cm from the tail. The incision was sutured, and a coating of cyanoacrylate glue was applied over the sutures. A 10 mg/ml bolus injection of haloperidol was given (250 µl subcutaneously near the pump modulator, and 250 µl i.p.). After recovering on a heating pad for at least 30 min the pup was returned to its home cage with the mother until use 16 h later. The dose of haloperidol and the method of administration was chosen in order to induce a pronounced catalepsy that was evident after the anesthesia wore off, and was still apparent after 16 h (data not shown).

2.8. Compounds

(RS)-3,5-Dihydroxyphenylglycine (DHPG), (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), and Methylphenylethynylpyridine (MPEP) were obtained from Tocris (Ballwin, MO). 4-[4-(*p*-Chlorophenyl)-4-hydroxypiperidino]-4'-flurobutyrophenone (Haloperidol) was obtained from Sigma (St. Louis, MO). Unless otherwise stated, all other materials were obtained from Sigma (St. Louis, MO).

3. Results

3.1. Colocalization of mGlu1 and mGlu5 to the same neurons in the STN and SNr

In our previous studies we have shown that the DHPG-induced depolarization of neurons in the STN and SNr is mediated solely by the activation of mGlu5 in the STN and mGlu1 in the SNr. These results were surprising, since in situ hybridization and immunocytochemical studies have shown that mRNA and protein for both mGlu1 and mGlu5 is present in the STN and SNr (Testa et al., 1994; Awad et al., 2000; Hubert et al., 2001). However, it has not been demonstrated that these receptors colocalize to the same cells. One possible explanation of our previous findings is that the mGlus are present in different cell populations within these nuclei, and that we selectively recorded from one of these cell types. To test this hypothesis, we performed a double labeling immunocytochemical study using confocal microscopy and fluorescent tagged secondary antibodies. As previously reported (Awad et al., 2000; Hubert et al., 2001) antibodies directed against mGlu1a or mGlu5 revealed a dense labeling of cells in both the STN and SNr (Figs. 1 and 2). Within the STN, 94% of the examined cells were immunopositive for mGlu1, and 90% were immunopositive for mGlu5. Interestingly, the mGlu5 immunofluorescence was most intense in the neuropil, with weaker labeling of the soma. The overlay of the two immunoprofiles (Fig. 1) demonstrates a high degree of colocalization of mGlu1 with mGlu5 within the STN. Of 50 cells examined, we found that 84% colocalized the two receptors. In the less cell-dense SNr, a similar pattern of labeling was apparent (Fig. 2). In this nucleus, 75% of the examined cells were immunopositive for mGlu1, while 89% were immunopositive for mGlu5. As demonstrated by the overlay in Fig. 2, 64% of cells in the SNr colocalize mGlu1 and mGlu5. From these experiments we can conclude that the majority of neurons within the STN and the SNr express both mGlu1 and mGlu5.

3.2. Both mGlu1 and mGlu5 play a role in the DHPG-induced increase in intracellular calcium

Since both mGlu1 and mGlu5 protein can be detected in the same neurons in the STN and the SNr, there must be an alternative explanation as to why only one of these receptors mediates the DHPG-induced depolarization observed in each of these regions. A recent report has demonstrated a differential subcellular localization of mGlu1 and mGlu5 at synapses in the SNr (Hubert et al., 2001). This study used immunogold localization of the receptors at the electron-microscopic level to demonstrate that <20% of the mGlu5 immunoreactivity observed in the SNr is associated with the membrane.



Fig. 1. Immunoreactivity of mGlula and mGlu5 in the STN. Individual cells within the STN labeled for mGlula (A) and mGlu5 (B). mGlul staining is prominent in the somatic cell membrane regions. There is also some punctate neuropil labeling apparent. The anti-mGlu5 antibody reveals intense staining of the neuropil with light somatic and cell membrane staining. (C) Overlay of (A) and (B) to demonstrate colocalization. Double labeling is observed in most cells, with a more prominent overlap of the two staining patterns near the plasma membrane and in the neuropil. Scale bar=50 µm.



Fig. 2. Immunoreactivity of mGlu1a and mGlu5 in the SNr. In the less cell dense SNr, individual cells can be observed that are labeled positively for mGlu1a (A), and mGlu5 (B). Intense staining is present within the cells and less so in the neuropil. (C) Overlay of A and B to demonstrate colocalization. While the majority of cells express both mGlu1a and mGlu5, the incidence of colocalization is lower than that observed in the STN. Scale bar=50 µm.

This raises the possibility that the differences we observed in group I mGlu pharmacology may be explained by differential subcellular localization. To test the hypothesis that one of the receptors was held in a nonmembrane bound, or inactive state, we employed calcium imaging using Fluo-3 as an indicator of intracellular calcium release.

Midbrain slices containing STN were bulk loaded with Fluo-3 as described in Materials and Methods. Brief (2 min) application of 50 µM DHPG induced a robust increase in fluorescence indicating an increase in intracellular calcium (Fig. 3). In order to determine which of the group I mGlus mediate the DHPG-induced increase in intracellular calcium, we employed recently developed antagonists that exhibit selectivity for either mGlu1 or mGlu5. LY367385 is a highly selective, competitive antagonist of mGlu1 with an IC50 in the low micromolar range that exhibits no effect on mGlu5 activation at concentrations up to 300 µM in recombinant and native systems (Clark et al., 1997; Awad et al., 2000; Mannaioni et al., 2001). MPEP is a highly selective, noncompetitive antagonist of mGlu5 with an IC50 in the nanomolar range that exhibits no effects on mGlu1 activation at concentrations up to 100 µM in recombinant or native systems (Gasparini et al., 1999; Mannaioni et al., 2001; Marino et al., 2001). Interestingly, either the mGlu1 selective antagonist LY367385 (300 µM) or the mGlu5 selective antagonist MPEP (10 µM) produced a significant block of the DHPG-induced increase in fluorescence $[F/F_{\Omega}]$ (488 nm) normalized to DHPG alone: DHPG=1.00±0.15 (n=21)cells): DHPG+LY367385=0.28±0.15 (n=18 cells), p<0.01, ttest: DHPG+MPEP= 0.20 ± 0.07 (n=20 cells), p<0.001, ttest]. Furthermore, the application of the combination of the two antagonists induced a further blockade of this response $[F/F_{O}]$ (488 nm): DHPG+LY367385+MPEP=0.04±0.03 (*n*=19 cells). p < 0.001, t-test]. This suggests that both mGlu1 and mGlu5 are present, play a role in the DHPG-induced increase in intracellular calcium, and are in a state that is accessible to the antagonists.

To perform similar experiments in the SNr, we could not rely on the bulk loading method used in STN. While the majority of neurons within the SNr are GABAergic, this nucleus represents a nonhomogeneous population of neurons making electrophysiological identification of GABAergic neurons a necessity. Therefore we performed similar experiments in the SNr by filling the cells with a Fluo-3 solution by diffusion from a whole cell patch pipette as described in Materials and Methods. 152

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Fig. 3. Both mGlu1 and mGlu5 play a role in the DHPG-induced increase in fluo-3 fluorescence in the STN. Representative records from individual cells (A), and images from selected fields of cells (B) demonstrating a robust increase in intracellular calcium levels induced by 50 μ M DHPG. White arrowheads indicate responsive soma. This response is partially sensitive to both the mGlu1-selective antagonist LY367386, and the mGlu5 antagonist MPEP. (C) Bar graph (mean±SEM) summarizing the partial block by either MPEP or LY367385, and the more complete block produced by the combination of the two antagonists (18–21 cells recorded from 4 to 5 slices for each condition; * p < 0.01, ** p < 0.001, t-test).

Under these conditions, brief (2 min) applications of 50 uM DHPG induced a robust increase in fluorescence in electrophysiologically identified GABAergic projection neurons (Fig. 4). Interestingly, similar to what was observed in the STN, application of either the mGlu1 selective antagonist LY367385 (300 μ M) or the mGlu5 selective antagonist MPEP (10 µM) produced a near complete block of the DHPG-induced calcium response $[F/F_{O}$ (488 nm) normalized to DHPG alone: DHPG=1.0±0.38 cells); (n=9 DHPG+LY367385=0.08±0.07 (n=4 cells), p<0.05, ttest; DHPG+MPEP=0.11±0.04 (n=6 cells), p<0.05, ttest; DHPG+LY+MPEP= 0.09 ± 0.04 (n=5 cells), p<0.05, t-test]. Taken together, these data suggest that in both the STN and SNr, mGlu1 and mGlu5 are present on the membrane and in an active state. Furthermore, this suggests that there must be some synergistic interactions between the two group I mGlus in these cells that enhances the induction of a calcium response.

3.3. Haloperidol treatment alters the pharmacology of the group I mGlu-mediated depolarization in the STN and SNr

If both mGlu1 and mGlu5 are colocalized to the same neurons and are in a functional, membrane bound state, then the different pharmacology observed in the STN and SNr must be due to some differential regulation of the coupling of these receptors to intracellular effector mechanisms. Since the group I mGlus interact with a

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Fig. 4. Both mGlu1 and mGlu5 play a role in the DHPG-induced increase in fluo-3 fluorescence in the SNr. Representative records from (A), and images (B) demonstrating a robust increase in intracellular calcium levels induced by 50 μ M DHPG in SNr GABAergic neurons loaded with fluo-3 by diffusion from the whole cell patch pipette. This response is blocked by both the mGlu1-selective antagonist LY367386, and the mGlu5 antagonist MPEP. (C) Bar graph (mean±SEM) summarizing the block by both MPEP and LY367385, and the lack of an additive effect of the combination of the two antagonists (n=4-9 cells/condition * p<0.05, *t*-test).

variety of intracellular regulatory proteins (for review see Blasi et al., 2001; Alagarsamy et al., 2001), it is possible that the coupling of these receptors is tightly regulated. If this is the case, it may also be possible that this type of regulation allows for some dynamic alteration in receptor coupling, and may in fact form the basis for a type of receptor plasticity in which the pharmacology of a given response may be determined by the state of the system. PD represents one particular state where the activity of the STN and the SNr are dramatically altered (Hollerman and Grace, 1992; Bergman et al., 1994; Hassani et al., 1996; Wichmann and DeLong, 1997). As a first approach to test for this type of dynamic regulation of receptor coupling, we utilized the dopamine receptor antagonist haloperidol to induce a prolonged (16 h) Parkinsonian state, and tested for differences in group I mGlu pharmacology in the STN and SNr.

As previously reported, and as shown in Fig. 5, the direct depolarizing effect of 50 μ M DHPG in STN neurons is completely blocked by the mGlu5-selective antagonist MPEP (10 μ M) and unaltered by application of the mGlu1-selective antagonist LY367385 (300 μ M). Interestingly, in slices made from animals that underwent a 16 h haloperidol treatment as described in



Fig. 5. Overnight haloperidol treatment alters the pharmacology of the group I mGlu-mediated depolarization in the STN. (A) In slices from control animals, application of 50 μ M DHPG induces a depolarization that is block by the mGlu5-selective antagonist MPEP, but not effected by preincubation with the mGlu1 antagonist LY367385. Sample traces (B) and mean±SEM data (C) illustrating the results of recordings in slices from animals that had undergone a 16 h haloperidol treatment to induce a prolonged Parkinsonian state demonstrating the consequent alteration in group I pharmacology. Note that in contrast to control recordings, preincubation with LY367385 now has a significant effect on the amplitude of depolarization and that a combination of MPEP and LY367385 is now required to fully block the effect of DHPG (4–7 cells per condition, * p < 0.05, ** p < 0.001, *t*-test).

Materials and Methods, the pharmacology of the group I mGlu-mediated depolarization was altered. In these slices from haloperidol-treated animals, MPEP (10 µM) did not induce a significant blockade of the DHPGinduced depolarization (Fig. 5B,C). In fact, LY367385 (300 μ M) produced a significant, but incomplete block of the DHPG-induced depolarization (DHPG=11.1±1.2 mV, n=6; DHPG+LY367385=5.7±1.87 mV, n=5, p<0.05, t-test; DHPG+MPEP=9.1±1.97 mV, n=7). A combination of these two antagonists was required in block the depolarization order to fully (DHPG+LY367385+MPEP=1.2±1.26 mV, n=4: p < 0.001), suggesting that in the post-haloperidol STN, the coupling of mGlu1 has been altered such that activation of this receptor is now capable of inducing a depolarization.

Similar experiments performed in the SNr demonstrate that, as previously reported, the DHPG-induced depolarization of SNr neurons is completely blocked by the mGlu1-selective antagonist LY367385 (300 μ M), and not affected by maximal concentrations of the mGlu5-selective antagonist MPEP (10 μ M) (Fig. 6A--C). Similar to what was observed in STN, there is a haloperidol-induced alteration in the coupling of mGlu5, such that post-haloperidol activation of mGlu1 or mGlu5 induces a depolarization. As shown in Fig. 6B,C, neither LY367385 (300 μ M) nor MPEP (10 μ M) are capable of blocking this response after haloperidol treatment (DHPG=25.7 \pm 7.1 mV, n=5; DHPG+LY367385=13.6 \pm 3.9 mV, n=8; DHPG+MPEP=15.0 \pm 7.6 mV, n=4). In fact, a combination of the two antagonists is required in order to induce a significant blockade of the response (DHPG+LY367385+MPEP=3.1 \pm 1.4 mV, n=4; p<0.05). Taken together, these data demonstrate a dynamic regulation of the pharmacology of group I mGlu-mediated depolarization in the STN and SNr.

4. Discussion

Previously, we have shown that while both mGlu1 and mGlu5 are present in neurons of the STN and SNr, there is a segregation of function of these closely related receptors. In the STN, DHPG induces a depolarization that is solely mediated by mGlu5. In contrast, activation of group I mGlus in the SNr induces a selective mGlu1mediated depolarization. Our current findings suggest that not only are both group I mGlus present in these two cell populations, but that they colocalize to the same neurons, and are in an active, membrane bound state. From this, we conclude that a regulatory mechanism exists that is capable of functionally segregating the two group I mGlus such that they couple to different effector systems. Interestingly, we have found that the receptor



Fig. 6. Overnight haloperidol treatment also alters the pharmacology of the group I mGlu-mediated depolarization in the SNr. (A) In slices from control animals, application of 50 μ M DHPG induces a depolarization that is blocked by the mGlu1-selective antagonist LY367385, but not effected by preincubation with the mGlu5 antagonist MPEP. Similar to the alteration observed in the STN, recordings in slices from animals that had undergone a 16 h haloperidol treatment to induce a prolonged Parkinsonian state revealed that this pharmacology had been altered (B, C) such that both mGlu1 and mGlu5 mediate the DHPG-induced depolarization after haloperidol. Bar graph represents mean±SEM data (*n*=4-8 cells/condition; * p < 0.05, *t*-test).

coupling in both the STN and SNr is somewhat plastic. Induction of a Parkinsonian state, a condition known to increase excitation of both the STN and the SNr (Hollerman and Grace, 1992; Bergman et al., 1994; Hassani et al., 1996; Wichmann and DeLong, 1997), alters the pharmacology of the group I mGlu-mediated depolarization in these nuclei, such that it is now produced by an activation of both mGlu1 and mGlu5.

At present, the molecular mechanisms that functionally segregate mGlu1 and mGlu5, and the mechanism underlying the change in receptor coupling observed after haloperidol treatment, are not clear. Potentially, the two receptors may be spatially segregated, and have differential access to signaling partners. This possibility is supported by recent immunogold studies examining the subcellular localization of the group I mGlus in the SNr (Hubert et al., 2001). This study observed that mGlu1a immunoreactivity is predominately associated with the membrane, whereas, >80% of mGlu5 immunoreactivity was localized to a cytoplasmic compartment. Recent data indicates that the membrane localization of group I mGlus is under the regulation of a family of Homer proteins that contain a PDZ-like protein interaction domain (Brakeman et al., 1997). These Homer proteins are believed to be involved in regulating the levels of receptor expression at the cell surface and in the positioning of these receptors close to second messengers necessary for signal transduction (Tu et al., 1998; Ciruela et al. 1999). Homer is known to differentially interact with the group I mGlus via a specific sequence found in the C-terminal portion of the receptor (Tu et al., 1998; Xiao et al., 1998). In particular, the expression of Homer 1a appears to regulate mGlu coupling (Tu et al., 1998; Kammermeier et al., 2000). Interestingly, Homer 1a is expressed as an immediate early gene, and is regulated by increases in cellular activity (Brakeman et al., 1997; Kato et al., 1997; Ango et al., 2000). This raises the intriguing possibility that increased activity in the STN and SNr during haloperidol treatment could induce the expression of Homer 1a and subsequent reorganization of the group I mGlus. Therefore, it is possible that under different physiological conditions, induction of the expression of receptor trafficking/localization proteins can change mGlu-effector coupling.

In addition to spatial segregation, it is also possible that a biochemical regulatory mechanism might explain the observed differences in mGlu1 and mGlu5 function as well as the haloperidol-induced alteration in coupling. The group I mGlus are modulated by a variety of intracellular effectors, any of which could potentially maintain either of the group I mGlus in a nonfunctional state. Protein kinase C (PKC) in particular plays important roles in the desensitization of group I mGlus (Schoepp and Johnson, 1988), and has been shown to directly phosphorylate mGlu5 thereby leading to its desensitiz-

ation (Gereau and Heinemann, 1998). Interestingly, mGlu1 undergoes a similar desensitization mechanism, in which PKC selectively desensitizes the IP₃/calcium release pathway by phosphorylating mGlu1a at particular sites in its C-terminal tail (Francesconi and Duvoisin, 2000). Thus, PKC can selectively desensitize one pathway activated by mGlu1 signaling, while leaving another pathway intact. In addition, activation of NMDA receptors has been shown to potentiate group I mGlumediated responses (Luthi et al., 1994; Alagarsamy et al., 1999) through activation of a calcium dependent phosphatase and reversal of PKC-mediated phosphorylation (Alagarsamy et al., 1999). If such a mechanism exists in neurons of the STN and SNr, activation of the NMDA receptor by increased release of glutamate in the Parkinsonian state might underlie the observed haloperidol-induced alteration in group I mGlu pharmacology. In addition to PKC, G-protein coupled receptor kinases (GRKs) (Dale et al., 2000; Sallese et al., 2000) and RGS proteins (regulators of G-protein signaling) (Saugstad et al., 1998; Kammermeier and Ikeda, 1999) have been demonstrated to regulate group I mGlu function.

There is increasing evidence indicating that dopaminergic denervation of the striatum in Parkinson's disease causes several regulatory changes in basal ganglia glutamatergic activity. Chronic D2 receptor blockade or dopaminergic denervation causes elevated extracellular levels of basal and potassium-releasable glutamate in the striatum (Calabresi et al., 1993; Yamamoto and Cooperman, 1994; Abarca et al., 1995). A unilateral dopamine denervation in the rat causes a downregulation of NMDA receptors, but not AMPA receptors, in the striatum, and the converse in the output nuclei, a downregulation of AMPA receptors, but not NMDA receptors (Porter et al., 1994). More recently, the GluR1 subunit of the AMPA receptor has been studied in more detail in MPTPinduced dopamine denervated primates and was found to be markedly increased in the striatum, and markedly decreased in the internal globus pallidus/SNr (Betarbet et al., 2000). Unilateral 6-hydroxydopamine (6-OHDA) lesions have been shown to cause a decrease in mGlu5 mRNA in the striatum compared to control (Yu et al., 2001) and an increase in mGlu3 and mGlu4 mRNA in the neostriatum and neocortex (Rodriguez-Puertas et al., 1999). These findings, combined with our data indicating a change in the pharmacology of the group I mGlu response in the STN and SNr, provide strong evidence that the glutamatergic system undergoes regulatory changes in response to altered basal ganglia activity in the parkinsonian state. For this reason, modulation of the glutamatergic system may provide a novel and beneficial approach for the treatment of PD.

While the underlying mechanism of regulation remains to be determined, the current study demonstrates a dynamic regulation of group I mGlus such that the receptor subtypes mediating the physiological actions of these receptors depends upon the state of the system. This is of particular importance for the hyperactivity of the STN and SNr observed in PD. While the hyperactivation of the STN has often been considered a consequence of disinhibition due to decreased pallidal activity (Miller and DeLong, 1987; Albin et al., 1989; Bergman et al., 1990; DeLong, 1990), it is now clear that glutamatergic input to the STN from the cortex plays an important role in regulation of this nucleus in both normal and pathological states (Hassani et al., 1996; Hirsch et al., 2000; Magill et al., 2000, 2001; Blandini et al., 2001). Furthermore, the increased excitatory drive through the indirect pathway and overexcitation of the SNr/EPN are the hallmark of PD, observed in both patients and in animal models of this disorder (Bergman et al., 1994; Benazzouz et al., 1996; Wichmann and DeLong, 1997). Recent studies have provided evidence that activation of group I mGlus could underlie, in part, the pathological excitation of the SNr, as activation of these receptors both directly depolarizes and disinhibits BG output. In particular, the prominent role of mGlu5 in directly exciting neurons in the STN, and facilitating a switch to burst firing has led to the suggestion that mGlu5-selective antagonists may provide a viable therapeutic option for the treatment of PD (Awad et al., 2000). However, when this hypothesis was tested using MPEP in the unilateral 6-OHDA model, researchers found only modest antiparkinsonian actions, (Spooren et al., 2000). This contrasts with a more recent study demonstrating clear antiparkinsonian actions of MPEP in the acute (1 h) haloperidol-induced catalepsy model (Ossowska et al., 2001). However, Spooren et al. (2000) noted that some interesting contradictions are apparent between the effects of MPEP in normal rats and in 6-OHDA lesioned rats, which led them to suggest that some plasticity may be occurring in response to this more chronic dopamine depletion. It is possible that the current findings demonstrating a haloperidol-induced alteration in group I mGlu pharmacology may help explain these discrepancies. However, it is important to note that while our model employs a more prolonged (16 h) haloperidol treatment, it is still relatively acute compared to a lesion model such as the 6-OHDA lesioned rat. Furthermore, while pharmacological blockade of dopamine receptors may induce a behavioral phenotype with many similarities to PD, it is clearly different from the slow neurodegenerative loss of dopamine that occurs in PD. Therefore, it will be important to confirm these findings in chronic models that more closely reflect the disease state. If the alterations in group I pharmacology observed in the present study occur in PD, this would have implications for the targeting of group I mGlus for novel therapeutics, and underscores the importance of performing target validation in an animal model that closely approximates the actual disease state.

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APPENDIX XIII

ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTOR 1 INHIBITS GLUTAMATERGIC TRANSMISSION IN THE SUBSTANTIA NIGRA PARS RETICULATA

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Abstract—The substantia nigra pars reticulata is a primary output nucleus of the basal ganglia motor circuit and is controlled by a fine balance between excitatory and inhibitory inputs. The major excitatory input to GABAergic neurons in the substantia nigra arises from glutamatergic neurons in the subthalamic nucleus, whereas inhibitory inputs arise mainly from the striatum and the globus pallidus. Anatomical studies revealed that metabotropic glutamate receptors (mGluRs) are highly expressed throughout the basal ganglia. Interestingly, mRNA for group I mGluRs are abundant in neurons of the subthalamic nucleus and the substantia nigra pars reticulata. Thus, it is possible that group I mGluRs play a role in the modulation of glutamatergic synaptic transmission at excitatory subthalamonigral synapses. To test this hypothesis, we investigated the effects of group I mGluR activation on excitatory synaptic transmission in putative GABAergic neurons in the substantia nigra pars reticulata using the whole cell patch clamp recording approach in slices of rat midbrain. We report that activation of group I mGluRs by the selective agonist (R,S)-3,5-dihydroxyphenylglycine (100 μ M) decreases synaptic transmission at excitatory synapses in the substantia nigra pars reticulata. This effect is selectively mediated by presynaptic activation of the group I mGluR subtype, mGluR1. Consistent with these data, electron microscopic immunocytochemical studies demonstrate the localization of mGluR1a at presynaptic sites in the rat substantia nigra pars reticulata.

From this finding that group I mGluRs modulate the major excitatory inputs to GABAergic neurons in the substantia nigra pars reticulata we suggest that these receptors may play an important role in basal ganglia functions. Studying this effect, therefore, provides new insights into the modulatory role of glutamate in basal ganglia output nuclei in physiological and pathophysiological conditions. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: basal ganglia, DHPG, movement disorders, presynaptic, subthalamic nucleus.

The basal ganglia is a highly interconnected group of subcortical nuclei in the vertebrate brain that plays a critical role in the control of movements. The GABAcontaining projection neurons of the substantia nigra pars reticulata (SNr) together with those of the entope-

duncular nucleus (EPN) are the main output nuclei of the basal ganglia (Grofova et al., 1982). GABAergic projection neurons in the SNr receive inputs from the striatum, the primary input nucleus of the basal ganglia, via two parallel but opposing pathways (DeLong, 1990; Bergman et al., 1990). The 'direct pathway' originates from a subpopulation of GABAergic striatal neurons that project directly to the SNr and, thereby, inhibit activity of these output neurons. The 'indirect pathway' originates from a different population of GABAergic striatal neurons that project to the SNr via the external segment of the globus pallidus (or globus pallidus in rats) and the subthalamic nucleus (STN). In turn, the STN provides excitatory glutamatergic inputs to the SNr. An intricate balance of activity between these pathways is believed to be necessary for a normal fine tuning of motor function, and the disruption of this balance leads to various movement disorders (Wichmann and DeLong, 1997, 1998). In Parkinson's disease (PD), the loss of nigrostriatal dopamine neurons results in a decreased activity of the direct pathway and an increased activity of the indirect pathway which leads to an increased firing

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Abbreviations: ACSF, artificial cerebrospinal fluid; DHPG, (R,S)-3,5-dihydroxyphenylglycine; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EPN, entopeduncular nucleus; EPSC, excitatory postsynaptic current; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); LY367385, (S)-(+)-α-amino-4-carboxy-2-methylbenzoacetic acid; mGluR, metabotropic glutamate receptor; MPEP, methylphenyl-ethynylpyridine; NMDA, N-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus.

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of STN neurons (Wichmann and DeLong, 1997). The overactive glutamatergic excitation of GABAergic neurons in the output nuclei of the basal ganglia (EPN/SNr) by the STN is believed to underlie the motor symptoms of PD (DeLong, 1990).

Glutamate is the major excitatory transmitter in the mammalian brain. While much effort has been focused on studying fast glutamatergic synaptic transmission via α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and *N*-methyl-D-aspartate (NMDA) receptors, recent studies indicate that glutamate also has important modulatory influences on excitatory synaptic transmission by action on metabotropic glutamate receptors (mGluRs).

These G protein-coupled receptors are highly expressed throughout the basal ganglia (Testa et al., 1994, 1998; Kerner et al., 1997; Kosinski et al., 1998, 1999; Bradley et al., 1999a,b). To date, eight mGluR subtypes (mGluR1-8) have been cloned, and are classified into three major groups based on sequence homology, coupling to second messenger systems, and selectivities for various agonists (Conn and Pin, 1997). Group I mGluRs (mGluR1 and 5) couple to G_q and activate phosphoinositide hydrolysis, while group II mGluRs (mGluR2 and 3) and group III mGluRs (mGluR4, 6, 7 and 8) couple to Gi/o and associated effector systems such as adenylyl cyclase. The mGluRs (with the exception of mGluR6) are widely distributed throughout the CNS and play important roles in regulating cell excitability and synaptic transmission at excitatory and inhibitory synapses (for review see Conn and Pin, 1997).

Previous anatomical studies have shown that both group I mGluR subtypes (mGluR1 and mGluR5) are present in STN and SNr neurons (Testa et al., 1994). Furthermore, recent studies in our laboratory have shown that activation of postsynaptically localized group I mGluRs produces a robust direct depolarization of putative projection neurons in both the SNr (Marino et al., 1999) and STN (Awad et al., 2000) in rats. Thus, group I mGluRs could play an important role in increasing the net excitatory drive onto SNr neurons and, thereby, increase the overall basal ganglia output. However, in addition to these postsynaptic effects, group I mGluRs in the hippocampus have also been shown to reduce excitatory and inhibitory synaptic transmission (Gereau and Conn, 1995; Manzoni and Bockaert, 1995). If group I mGluRs have similar effects in the SNr, this could influence the overall impact of group I mGluR activation on transmission at STN-SNr synapses. To investigate this issue, we performed a series of in vitro whole cell patch clamp recording studies to determine whether activation of group I mGluRs modulates excitatory glutamatergic transmission in SNr neurons.

EXPERIMENTAL PROCEDURES

Materials

[*R*-(*R**,*S**)]-6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5-g]isoquinolin-5-yl)furo[3,4-e]-1,3-benzodioxol-8(6*H*)-one (bicuculline), 7-hydroxyiminocyclopropan-[*b*]chromen-1a-carboxylic acid ethyl ester, (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG), (*S*)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) and methyl-phenyl-ethynylpyridine (MPEP) were obtained from Tocris (Ballwin, MO, USA). All other materials were obtained from Sigma (St. Louis, MO, USA).

Electrophysiology

Whole cell patch clamp recordings were obtained under visual control as previously described (Marino et al., 1998; Bradley et al., 2000). Fifteen- to eighteen-day-old Sprague-Dawley rats (Charles River, USA) were used for all patch clamp studies. The animals were anesthetized with chloral hydrate (700 mg/ kg) and transcardially perfused with an ice cold sucrose buffer (in mM: sucrose, 187; KCl, 3; MgSO₄, 1.9; KH₂PO₄, 1.2; glucose, 20; NaHCO₃, 26; equilibrated with 95% O₂/5% CO₂). Brains were rapidly removed and submerged in ice cold sucrose buffer. Parasagittal slices (300 µm thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber containing normal artificial cerebrospinal fluid (ACSF; in mM: NaCl, 124; KCl, 2.5; MgSO₄, 1.3; NaH₂PO₄, 1.0; CaCl₂, 2.0, glucose, 20; NaHCO₃, 26; equilibrated with 95% $O_2/5\%$ CO₂). In all experiments, 5 μ M glutathione and 500 μ M pyruvate were included in the sucrose buffer and holding chamber. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room temperature ACSF (~3 ml/min, 23-24°C). Neurons in the SNr were visualized with a $40 \times$ water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with a mixture of (in mM) potassium gluconate, 140; HEPES, 10; NaCl, 10; EGTA, 0.6; NaGTP, 0.2; MgATP, 2; pH adjusted to 7.4 with 0.5 N KOH. Electrode resistance was 3-7 MΩ.

GABAergic SNr neurons were identified according to previously established electrophysiological criteria (Richards et al., 1997). GABAergic neurons exhibit spontaneous repetitive firing, short duration action potentials, little spike frequency adaptation, and a lack of inward rectification, whereas dopaminergic neurons display no or low frequency spontaneous firing, longer duration action potentials, strong spike frequency adaptation, and a pronounced inward rectification. All data presented in this study are from neurons which fit the electrophysiological criteria of GABAergic neurons.

Excitatory postsynaptic currents (EPSCs) were evoked with bipolar tungsten electrodes (0.4–1.2 μ A every 30 s). The stimulation electrode was placed into the STN or the cerebral peduncle rostral to and outside the SNr. EPSCs were recorded from a holding potential of -60 mV. Bicuculline (10 μ M) was bath applied during all EPSC recordings to block inhibitory transmission.

Values are expressed as mean \pm S.E.M. Statistical significance was assessed using Student's *t*-test. P < 0.05 was set as the limit of statistical significance.

Immunohistochemical method

Animal perfusion and preparation of tissue. Three male Sprague-Dawley rats, 14 days old, were deeply anesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with cold, oxygenated Ringer's solution followed by 100 ml of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) and 50 ml of cold PB. Next, the brain was removed from the skull and stored in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) before being sliced on a vibrating microtome into 60-µm transverse sections. These sections were then treated with 1.0% sodium borohydride for 20 min and rinsed in PBS.

Electron microscope immunohistochemistry. For electron microscopy studies, the sections were treated with cryoprotectant for 20 min and transferred to a -80° C freezer for an additional 20 min, returned to a decreasing gradient of cryoprotectant solutions and rinsed in PBS. Sections then underwent immunocytochemical procedures for the immunoperoxidase

localization of mGluR1a. The sections were preincubated at room temperature in a solution containing 10% normal horse serum and 1.0% bovine serum albumin in PBS for 1 h. They were then incubated for 48 h at 4°C in a solution containing a monoclonal antibody raised against the C-terminus of mGluR1a (Pharmingen, San Diego, CA, USA) diluted at 0.25 µg/ml in the preincubation solution. This antibody has been shown to be highly specific for mGluR1a in both immunoblot and immunocytochemical studies performed on rat tissue and transfected HEK cells. Furthermore, this staining is blocked by preadsorption with the antigenic peptide (Shigemoto et al., 1994; Petralia et al., 1997). Next, the sections were rinsed in PBS and transferred for 1 h at room temperature to a secondary antibody solution containing biotinylated horse anti-mouse IgGs (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in the primary antibody diluent solution. After rinsing, sections were put in a solution containing 1:100 avidin-biotin-peroxidase complex (Vector). The tissue was then washed in PBS and 0.05 M Tris buffer before being transferred to a solution containing 0.01 M imidazole, 0.0005% hydrogen peroxide, and 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) in Tris for 7-10 min. The sections were then transferred to PB (0.1 M, pH 7.4) for 10 min and exposed to 1% osmium tetroxide for 20 min. Afterwards, they were rinsed with PB and dehydrated in an increasing gradient of ethanol. Uranyl acetate (1%) was added to the 70% alcohol to increase contrast at the electron microscope. The sections were then treated with propylene oxide before being embedded in epoxy resin (Durcupan, ACM, Fluka, Buchs, Switzerland) for 12 h, mounted on microscope slides and placed in a 60°C oven for 48 h.

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One block of SNr tissue was taken from each rat and glued on the top of resin blocks with cyanoacrylate glue. They were cut into 60-nm ultrathin sections with an ultramicrotome (Ultracut T2, Leica, Nussloch, Germany) and serially collected on single-slot Pioloform-coated copper grids. The sections were stained with lead citrate for 5 min and examined with a Zeiss EM-10C electron microscope (Thornwood, NY, USA). Electron micrographs were taken at low and high magnifications to characterize the nature of mGluR la-immunoreactive elements in the SNr.

The anesthesia and euthanasia procedures were carried out according to the National Institutes of Health Guidelines and have been accepted by the Institutional Animal Care and Use Committee of Emory University. All efforts were made to reduce the number of animals used and to minimize animal suffering.

RESULTS

Whole cell patch clamp experiments were performed at electrophysiologically identified GABAergic neurons of the SNr in midbrain slices. EPSCs were elicited by stimulation of the STN or the cerebral peduncle, rostral to the SNr with bipolar stimulation electrodes. We have previously demonstrated that EPSCs elicited by STN stimulation with this protocol are of a constant latency and blocked by 6-cyano-7-nitroquinoxaline-2,3-dione



Fig. 1. Application of DHPG suppresses EPSCs in SNr GABAergic neurons. (A) Example traces of evoked EPSCs before (Pre-Drug), during (DHPG) and after (Washout) brief bath application of 100 μ M DHPG. (B) Average time course of the effect of 100 μ M DHPG demonstrating that the effect of DHPG is reversible. Values were normalized to a Pre-Drug baseline. Each point represents the mean (±S.E.M.) of data from six cells. (C) Dose-response relationship of the effect of DHPG on EPSC amplitudes showing that the effect of DHPG is dose-dependent with an EC₅₀ of about 5 μ M. Each point represents the mean (±S.E.M.) of data from four to six cells.



Fig. 2. The effect of DHPG on EPSCs is mediated by mGluR1 but not mGluR5. (A-C) Example traces showing the effects of mGluR1 and mGluR5 selective antagonists on the 50 μM DHPG-induced effects on EPSCs. Antagonists include the mGluR5 selective, non-competitive antagonist MPEP 10 μM (B) and the mGluR1 selective, competitive antagonist LY367385 300 μM (C). (D) Bar graph showing the average effect of mGluR1 and mGluR5 selective antagonists on the DHPG-induced effect. Each bar represents the mean (±S.E.M.) of data collected from three to five cells (*P<0.05, t-test).

disodium (CNQX) suggesting that they are monosynaptic and mediated by glutamate (Bradley et al., 2000). Since there was no significant difference between results obtained with STN stimulation and results obtained with peduncle stimulation, data from the two sets of experiments were pooled in this study.

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Activation of group I mGluR inhibits excitatory synaptic transmission in SNr neurons

All recordings were performed at a holding potential of -60 mV in the presence of bicuculline (10 μ M) to block inhibitory synaptic transmission.

Brief bath application of the group I mGluR selective agonist DHPG (100 μ M) produced a significant depression of EPSCs in GABAergic SNr neurons (Fig. 1A; P < 0.05; n = 6). This effect of DHPG was reversible (Fig. 1B). The concentration-response relationship for the DHPG-induced depression of EPSCs in SNr neurons revealed an EC₅₀ around 5 μ M with a maximal effect of 44.5±2.5% at a concentration of 100 μ M DHPG (n = 6, Fig. 1C). This is a concentration consistent with an effect on group I mGluRs (Schoepp et al., 1994).

Taken together, these data suggest that activation of group I mGluRs reduces excitatory synaptic transmission in the SNr. Since both group I mGluR subtypes, mGluR1 and mGluR5, are present in STN and SNr neurons (Testa et al., 1994), we used newly available pharmacological tools that distinguish between mGluR1 and mGluR5 to determine which receptor subtype mediates the depression of excitatory transmission in the SNr. Prior application (10-15 min) of MPEP $(10 \ \mu\text{M})$, a highly selective, non-competitive antagonist of mGluR5, showed no significant effect in blocking the effect of 50 μ M DHPG on excitatory synaptic transmission in the SNr at concentrations shown to be effective at blocking mGluR5 effects in other systems (Gasparini et al., 1999; Bowes et al., 1999) and postsynaptic mGluR5 in both STN and SNr (Marino et al., 1999; Awad et al., 2000) (Fig. 2B,D). The mGluR1 selective, competitive antagonist LY367385 (300 μ M) (Clark et al., 1997), in contrast, completely blocked the effect of 50 μ M DHPG on excitatory synaptic transmission in the SNr (Fig. 2C,D). Taken together, these data suggest that activation of mGluR1 reduces glutamatergic synaptic transmission in the SNr.

The inhibitory effect of mGluR1 activation on synaptic transmission is mediated by a presynaptic mechanism

To test the hypothesis that this effect is mediated by presynaptic mechanisms we determined the effect of DHPG on paired-pulse facilitation of evoked EPSCs. All paired-pulse recordings were performed at a holding potential of -60 mV in the presence of bicuculline (10 μ M) and EPSCs were evoked by stimulating the cerebral peduncle every 20 s by paired stimulations of equal strength at 20-100-ms intervals. Stimulus strength and inter-pulse intervals were adjusted in each experiment so that the second EPSC was always greater in amplitude than the first (paired-pulse facilitation: $139.8 \pm 9.8\%$, n = 5). DHPG (100 μ M) reduced the absolute amplitude



Fig. 3. Activation of the group I mGluR subtype, mGluR1, increases the ratio of paired-pulse facilitation of evoked EPSCs. (A, B) Representative traces of paired-pulse facilitation before (Pre-Drug) and during application of 100 μ M DHPG. (C) Superimposed traces of pre-drug condition and during application of DHPG (trace scaled to first EPSC of control condition). (D) Bar graph showing the average effect of DHPG on the ratio of paired-pulse facilitation. Each bar represents the mean (± S.E.M.) collected from five cells (*P < 0.01; *t*-test).

of EPSCs but also increased the ratio of paired-pulse facilitation significantly to $260.4 \pm 37.4\%$ (Fig. 3, P < 0.01, n=5, *t*-test). This represents an $88.9 \pm 28.5\%$ increase of facilitation induced by DHPG. Taken together, these data provide strong support for the hypothesis that DHPG acts presynaptically to inhibit the evoked release of transmitter from glutamatergic terminals.

Presynaptic localization of mGluR1a in the SNr

In order to confirm the presence of presynaptic mGluR1a in the SNr, we used a monoclonal antibody to immunohistochemically localize mGluR1a at the electron microscope level. Consistent with previous findings, dendritic elements of various sizes were strongly immunoreactive (Fig. 4A, B) (Hubert and Smith, 1999). In addition to postsynaptic elements, numerous unmyelinated axons and a few axon terminals forming asymmetric synapses were immunolabeled (Fig. 4A-C). While the identity of the small unmyelinated axons cannot be definitively determined, these structures are reminiscent of what would be expected for preterminal axonal segments. This, coupled with the presence of mGluR1a on terminals forming asymmetric synapses, suggests that mGluR1a is localized on presynaptic elements of excitatory synapses.

DISCUSSION

The data presented in this study reveal that activation of group I mGluRs reduces glutamatergic synaptic transmission in GABAergic SNr neurons and that this effect is mediated by a presynaptic mechanism.

We have previously shown that stimulation of the STN with the protocol used in this study induces glutamatergic EPSCs in SNr neurons (Bradley et al., 2000). In addition, since a large percentage of excitatory terminals on SNr GABAergic neurons arise from the STN (Smith et al., 1998), it is very likely that the observed inhibitory effect of DHPG on glutamatergic synaptic transmission is mediated by action on group I mGluRs localized at STN-SNr synapses. However, since the STN is not the only source of asymmetric synapses observed in the SNr, effects on other glutamatergic synapses cannot be excluded.

Our immunocytochemical studies suggest that the group I mGluR subtype mGluR1a is localized on unmyelinated axons in the SNr and is also found presynaptic in a few terminals forming asymmetric synapses. This is in agreement with our pharmacological studies showing that the inhibition of EPSCs induced by DHPG is solely mediated by the subtype mGluR1 but not mGluR5. Furthermore, our findings that DHPG



Fig. 4. Immunoperoxidase mGluR1a labeling in the rat SNr at the electron microscope level. (A) mGluR1a-immunoreactive terminal (Ter) forming an asymmetric synapse (arrowheads) with an immunoreactive dendrite (Den). (B) mGluR1a-immuno-reactive terminal in the monkey SNr apposed to an mGluR1a-immunoreactive dendrite. Due to the dense peroxidase deposit, the synaptic specialization cannot be visualized. Note that the preterminal portion of the axon (Ax) is also strongly immuno-reactive. (C) mGluR1a-immunoreactive ummyelinated axons in the SNr. Scale bar = 1.0 μm.

increases the ratio of paired-pulse facilitation indicates that this effect is mediated by a presynaptic mechanism. Both group I mGluR subtypes, mGluR1 and mGluR5, are expressed in STN neurons (Awad et al., 2000). Despite the coexistence of the two mGluR subtypes, a recent *in vitro* study shows that DHPG-induced depolarization and potentiation of postsynaptic NMDA receptor currents in STN neurons are exclusively mediated by the mGluR5 subtype (Awad et al., 2000). This suggests that there might exist a difference in trafficking for the two group I mGluR subtypes in STN neurons. While the subtype mGluR5 is mainly localized postsynaptically in dendrites of STN neurons, the subtype mGluR1 could be trafficked to STN terminals, acting as a presynaptic autoreceptor. Taken together, our data indicate that DHPG decreases synaptic transmission at STN-SNr synapses by activation of the presynaptically localized group I mGluR subtype mGluR1.

However, it should be noted that other mechanisms might mediate the effects of group I mGluR activation on EPSCs. For instance, studies in hippocampal CA1 pyramidal cells (Alger et al., 1996; Morishita et al., 1998: Morishita and Alger, 1999) and cerebellar Purkinje cells (Llano et al., 1991; Vincent et al., 1992; Vincent and Marty, 1993) suggest that depolarization-induced release of an unknown retrograde messenger can reduce inhibitory synaptic transmission by acting at presynaptic sites on GABAergic terminals. This so-called depolarization-induced suppression of inhibition involves a transient (~1 min) suppression of GABAA receptor-mediated inhibitory postsynaptic currents impinging on these cells after depolarization of their membranes that is sufficient to open voltage-gated Ca²⁺ channels. We have previously shown that activation of mGluR1 induces a robust depolarization of GABAergic SNr neurons by acting on postsynaptically localized receptors (Marino et al., 1999. 2000). This raises the possibility that the effect of DHPG on excitatory synaptic transmission might be induced by a postsynaptic mGluR1-mediated depolarization of SNr neurons and subsequent release of a retrograde messenger which, then, acts on presynaptic sites in glutamatergic axons and terminals.

Although the most common role of group I mGluRs is the postsynaptic regulation of neuronal excitability, activation of group I mGluRs has been shown to decrease glutamate release in other brain regions, including the CA1 region of the hippocampus (Gereau and Conn, 1995; Manzoni and Bockaert, 1995). Activation of presynaptic mGluRs can also facilitate glutamate release (Herrero et al., 1992) probably due to diacylglycerol production and protein kinase C activation (Herrero et al., 1994; Coffey et al., 1994). Interestingly, recent electrophysiological and biochemical studies in hippocampus and cerebral cortex suggest that presynaptically localized group I mGluRs undergo an activity-dependent switch where activation causes, first, a facilitation of synaptic transmission and then a depression (Herrero et al., 1998; Rodriguez-Moreno et al., 1998). It has been suggested that this is mediated by desensitization of signaling pathways involved in facilitation of release so that inhibition of release predominates after prolonged agonist application. We did not see evidence of this biphasic effect on EPSCs in the SNr. However, detection of the facilitatory phase in hippocampus requires relatively rapid agonist application that was not used in the present study. It is noteworthy that even the increased ambient concentration of extracellular glutamate due to slice preparation may induce the desensitization of the facilitatory response, switching the receptor function to inhibition in hippocampal slices (Rodriguez-Moreno et al., 1998). It is therefore possible that, in our slices, the extracellular glutamate concentration is high enough to act on presynaptic group I mGluRs thereby inducing a

switch of the receptor state towards inhibition of glutamate release.

Our current findings add to a growing body of literature suggesting that group I mGluRs play an important role in regulating basal ganglia functions. Both group I mGluR subtypes are expressed throughout the basal ganglia (Testa et al., 1995; Kerner et al., 1997; Kosinski et al., 1998; Hanson and Smith, 1999; Smith et al., 2000) and have been shown to modulate neuronal activity in various basal ganglia structures. For instance, activation of group I mGluRs potentiates NMDA receptor currents in striatal medium spiny neurons (Colwell and Levine, 1994; Pisani et al., 1997). Furthermore, behavioral studies combined with measurements of changes in 2-deoxyglucose uptake and c-fos expression suggest that injection of group I mGluR agonists in the striatum selectively increases transmission through the indirect pathway (Kaatz and Albin, 1995; Kearney et al., 1997). Previous anatomical studies showed that neurons in the STN express both group I mGluR subtypes (Testa et al., 1994, 1998; Awad et al., 2000). In line with these findings, recent electrophysiological data demonstrated that activation of group I mGluRs induces a robust depolarization and potentiates NMDA receptor currents in STN neurons (Awad et al., 2000). Interestingly this effect is selectively mediated by activation of the group I mGluR subtype mGluR5. Group I mGluRs have also been shown to increase cell excitability and potentiate NMDA receptor currents in the rat SNr (Marino et al., 1999, 2000). In contrast to STN neurons this effect is solely mediated by activation of the subtype mGluR1. Additionally, group I mGluRs decrease inhibitory synaptic transmission in GABAergic SNr neurons (Wittmann et al., 2000). Our finding that activation of mGluR1 decreases glutamate release in the SNr seems to be opposing the effects of released glutamate on postsynaptic group I mGluRs. However, it is conceivable that these opposing effects of glutamate at pre- and postsynaptic sites act in concert to provide a filter mechanism that increases the signal to noise ratio of transmission at STN-SNr synapses. While postsynaptic activation produces an increase in excitability (direct depolarization) and sensitivity to glutamate (potentiation of NMDA receptor currents) (Marino et al., 2000), presynaptic activation reduces synaptic transmission, thereby ensuring that only strong signals elicit a significant postsynaptic response. Because of the postsynaptic actions, these stronger signals would, then, have a larger effect on activity of neurons in the SNr. Future studies at the circuit and systems level will be required to gain a complete understanding of the overall impact of group I mGluR activation on transmission through the basal ganglia circuits.

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APPENDIX XIV

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Distinct Functional Roles of the Metabotropic Glutamate Receptors 1 and 5 in the Rat Globus Pallidus

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ABSTRACT

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Group I metabotropic glutamate receptors 1 and 5 frequently co-localize in the same neurons throughout the central nervous system. Since both receptors can couple to the same effector systems, the purpose of their cellular co-expression remains unclear. Here, we report that group I metabotropic glutamate receptors (mGluRs) 1 and 5 have distinct functional roles in type II neurons of the rat globus pallidus (GP). Type II GP neurons form a large population of GABAergic projection neurons that are characterized by the presence of inwardly rectifying current I_h, low threshold voltage-activated calcium current It, and activity at rest. Although immunocytochemical analysis reveals a high degree of neuronal co-localization of the two group I mGluRs in the GP, activation of mGluR1 only directly depolarizes type II GP neurons. Interestingly, blockade of mGluR5 by a highly selective antagonist, MPEP, leads to the potentiation of the mGluR1mediated depolarization in this neuronal sub-population. Metabotropic GluR1 desensitizes upon repeated activation with the agonist in type II GP neurons and blocking mGluR5 prevents the desensitization of the mGluR1-mediated depolarization. Elimination of the activity of protein kinase C (PKC) by an application of 1µM bisendolylmaleimide or 1 µM chelerythrine, both protein kinase C inhibitors, potentiates the mGluR1-mediated response and prevents the desensitization of mGluR1 in type II GP neurons, suggesting that the effect of mGluR5 on mGluR1 signaling may involve PKC. Together, these data illustrate a novel mechanism by which mGluR1 and mGluR5, members of the same family of G-protein coupled receptors, can interact to modulate neuronal activity in the rat GP.

INTRODUCTION

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Eight metabotropic glutamate receptors (mGluRs) have been cloned thus far and they have been sub-divided into three groups based on sequence homology, agonist selectivity, and coupling to specific second messenger cascades. The metabotropic glutamate receptors 1 and 5 belong to the group I mGluRs. There are many similarities in the effector systems activated by either receptor (for review, see Hermans, 2001). Classically, both mGluR1 and mGluR5 are known to activate phospholipase C via coupling to $G_{q/11}$ proteins, which leads to intracellular Ca^{2+} release and activation of protein kinase C (PKC) (for review, see Conn, 1994). In turn, PKC can negatively feedback on the group I mGluR signaling by phosphorylation of mGluR1 (Francesconi and Duvoisin, 2000) and mGluR5 (Gereau and Heinemann, 1998), which leads to the receptor desensitization (Alagarsamy et al., 1999; Kawabata et al., 1996). Desensitization of group I mGluRs can also occur via proteins that regulate G-protein signaling (RGS), protein kinase A (PKA) or G protein-coupled receptor kinases (GRKs) (Sallese et al., 2000; for review, see Alagarsamy et al., 2001 or De Blasi, 2001).

Despite many similarities in the effector systems that are activated by mGluR1 or mGluR5, it is becoming increasingly clear with the introduction of sub-type selective antagonists that mGluR1 and mGluR5 fulfill distinct functional roles whenever they co-exist in the same neurons. (Mannaioni et al., 2001; Calabresi et. al., 2001; Gubellini et al., 2001; Pisani et al., 2001b; and for review see Valenti et al., 2002).

Group I mGluRs are both present in the globus pallidus (GP), the subthalamic nucleus (STN), and substantia nigra pars reticulata (SNr) and the striatum (Hanson and Smith, 1999; Awad et al., 2000; Marino et al., 2001b; Tallaksen-Greene et al., 1998; and

for review, see Rouse et al., 2000). Metabotropic GluR1 and mGluR5 carry distinct functions in the STN, SNr and the striatum where they co-localize to the same neurons (Marino and Conn, 2001a, Pisani et al., 2001a). However, functional roles of these receptors in the GP, a component of the so-called "indirect pathway" of the BG, have been largely unexplored.

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GP neurons are GABAergic and heterogeneous in morphology and physiological criteria gathered from in vitro slice preparations. A consensus from many studies is that type A, also referred to as type II neurons (Nambu and Llinas, 1994; Stanford and Cooper, 1999; Shindou et al., 2001) are the predominant electrophysiological phenotype in the rodent GP (Cooper and Stanford, 2000). Here, we report that activation of mGluR1, but not mGluR5, depolarizes type II GP neurons. However, blockade of mGluR5 potentiates the mGluR1-mediated response to stimulation by preventing the desensitization of mGluR1 in these neurons. The potentiation of the mGluR1-mediated depolarization and the prevention of the desensitization of mGluR1 are both mimicked by the blockade of PKC. Our data provide evidence for the functional specificity of mGluR1 and mGluR5 when co-expressed in the same neurons and reveal a novel mode of functional interaction between the group I mGluRs in the CNS.

MATERIALS AND METHODS

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Materials. (RS)-3,5-dihydroxyphenylglycine (DHPG), L(+)-2-amino-4phosphonobutyric acid (L-AP4), (S)-(+)-a-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), methylphenylethynylpyridine (MPEP), were obtained from Tocris Cookson (Ballwin, MO). (+)-2-Aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740) was a gift from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). Bisendolylmaleimide I, HCl (Bis), and chelerythrine chloride (Chel) were obtained from Calbiochem (Cambridge, MA). Phorbol 12-myristate 13-acetate (PMA), 4-α- phorbol 12myristate 13-acetate (4-α-PMA), tetrodotoxin (TTX) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

<u>Group I mGluRs Immunocytochemistry</u>. All animal work was performed in accordance with Emory University IACUC protocols and procedures. Two 15 days old Sprague-Dawley rats were anesthetized with isoflurane and transcardially perfused with normal saline, which was supplemented with 0.005% sodium nitroprusside. Saline was followed by a ten-minute perfusion with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (PB) (0.1M, pH 7.4). The brains were then removed and post-fixed in the same fixative overnight at 4°C. Fifty µm-thick sections were cut in cold PB on OTS-4000 Tissue Slicer (Frederick Haer and Co., Bowdoinham, ME). Before processing for immunocytochemistry, sections were stored in a mixture of 30% sucrose and 30% ethylene glycol in PB at -20° C.

All incubations for the immunocytochemistry were performed at room temperature and all washes were done with PB. Sections were washed and incubated for 10 minutes with 3% hydrogen peroxide/PB solution. After another wash, sections were pre-incubated for 30 minutes with a mixture of avidin (10 µg/ml), 5% normal goat serum and 5% normal horse serum in PB. Sections were again washed with PB and incubated overnight with a cocktail of antibodies, raised against mGluR1a (mouse monoclonal, PharMingen, San Diego, CA) and mGluR5 (rabbit polyclonal, Upstate Biotechnologies, Lake Placid, NY). Specificity of these antibodies was demonstrated in previous study (Marino et al., 2001a). Metabotropic GluR1a and mGluR5 antibodies were diluted 1:2,000 and 1:1,000, respectively, in a mixture of avidin (50 µg/ml), 1% normal goat and 1% normal horse sera in PB. Sections were then washed and co-incubated for 1 hour with donkey anti-rabbit IgGs (1:100) conjugated to rhodamine and biotinylated goat antimouse IgGs (1:100). Both secondary antibodies were obtained from Jackson Labs, Bal Harbor, MN. Sections were again washed. Metabotropic GluR1a staining was further amplified with ABC (1:500, 30 minutes, Vector Labs, Burlingame, CA) followed by tyramide conjugated to FITC (1:100, 10 minutes, Perkin Elmer Life Sciences, Boston, MA). After another wash and incubation for 30 minutes with a mixture of 10 mM cupric sulfate and 50 mM ammonium acetate (pH 5.0), sections were wet mounted on superfrost plus glass slides (Fisher Scientific, Atlanta, GA) and coverslipped with Vector Vectashield mounting medium. Slides were always stored in the dark at 4°C. Metabotropic GluR1a and mGluR5 staining in the GP was visualized on a Zeiss confocal microscope and acquired images were processed using Adobe PhotoShop software (San Jose, CA).

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In control experiments, each primary antibody was omitted in turn while the rest of the double-labeling procedure remained the same. This led to labeling for only one receptor subtype, which indicates that there was no cross-reactivity between secondary antibodies in the double-labeling procedure.

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<u>Biocytin Histochemistry</u>: To visualize biocytin-filled GP neurons, slices were incubated at room temperature in 10 % paraformaldehyde overnight. Slices &ere then washed with PB and pre-incubated with a mixture of 1% hydrogen peroxide, 10% methanol, and 2% albumin in PB for 30 min at RT. The pre-incubation was followed by washes in PB and an overnight incubation at 4°C with Vector ABC solution diluted in 0.1% TritonX-100 and 2% Albumin in PB. Slices were washed again with PB and incubated for about 10 min with Vector SG Chromagen. Slices were then washed with PB and wet mounted on Fisher superfrost plus slides. Sections were then allowed to dry overnight at room temperature and dehydrated by sequential incubations in 70%, 90%, 100% ethanol and xylene before being coverslipped with Permount and viewed using a Hoffmann modulation contrast microscope and processed using Adobe PhotoShop software.

Slice Preparation and Electrophysiology. All whole-cell patch-clamp recordings were obtained as previously described (Bradley et al., 2000; Marino et al., 1998). Fourteeneitghteen days-old Sprague-Dawley rats were used in all experiments. After decapitation, brains were removed and quickly submerged in the ice-cold oxygenated sucrose buffer (in mM: 223.4 sucrose, 20 glucose, 47.3 NaHCO₃, 3 KCl, 1.9 MgSO₄, 1.2 KH₂PO₄, 2 CaCl₂), which was always supplemented with sodium pyruvate (80 µM) and glutathione

(0.78 µM). Parasagittal slices (250 or 300 µm-thick) were made on a tissue slicer (World Precision Instruments, Sarasota, FL) in ice-cold oxygenated sucrose buffer. Slices were transferred into a holding chamber containing normal ACSF (in mM: 124 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 2.0 CaCl₂), which was continuously bubbled with 95% O₂-5% CO₂ gas mixture. The osmolarity of the ACSF was around 330 mOsm. ACSF in the holding chamber was always supplemented with sodium pyruvate (0.125 mM), glutathione (0.0012 mM), and kynurenic acid (0.06 mM). These additives tended to increase slice viability and had no effect on experiments. In two experiments we found that omission of sodium pyruvate, glutathione, and kynurenic acid from the ACSF or the sucrose solution did not alter the DHPG-induced effect on the membrane potential in type II GP neurons. We therefore included these results in our DHPG pool in Figures 4B, 4E, 7B, and 7D. GP neurons were visualized with a 40X water immersion lens using a Hoffman modulation contrast microscope. Slices were continuously perfused with room temperature oxygenated ACSF. Borosilicate glass patch electrodes were pulled on a vertical patch pipette puller (Narashige, Tokyo, Japan) and filled with an intracellular patch solution (in mM: 140 potassium gluconate, 16 HEPES, 10 NaCl, 2 EGTA, 2, MgATP, 0.2 NaGTP), pH 7.5. Biocytin at 0.5% was sometimes included in the intracellular solution to permit post-hoc analysis of morphology and location of GP neurons. Bis (1 μ M), Chel (1 μ M), PMA (10 or 100 nM), or 4- α -PMA (100 nM) was included in the intracellular patch solution in experiments where the role of PKC in the function of mGluR1 was evaluated. The osmolarity of the intracellular solution was always adjusted to about 310 mOsm. All neurons were visually classified into two types based on electrophysiological criteria described in the Results section. If a neuron did not

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fit into either type, it was discarded before an experiment began. Series resistance (20-30 M Ω) was recorded at the beginning and at the end of each experiment and an experiment was discarded if the series resistance changed by more than 20%. Ten pA hyperpolarizing current injections were given intermittently throughout each experiment to monitor the effect of agonists/antagonists on input resistance. Slices were perfused with TTX (0.5 μ M) for at least 5 min before the commencement of all experiments.

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IV relationship. Electrodes were filled with (in mM) 140 potassium gluconate, 16 HEPES, 10 NaCl, 2 EGTA, 2 MgATP, and 0.2 NaGTP. Standard ACSF was used with addition of: TTX (1 μ M), bicuculline (10 μ M), CNQX (25 μ M) and APV (50 μ M). Depolarizing pulses (-10 mV amplitude and 40 ms long) were periodically applied to monitor membrane conductance and a chart recorder was used to monitor the holding current. The IV relationship was assessed by ramping the membrane potential from +10 mV to -130 mV (20 mV/s) prior to drug application and at the time of maximal DHPGinduced inward current. Voltage-dependent calcium currents were inactivated by holding the membrane potential at + 10 mV for 1 sec prior to initiating the ramp.

<u>Data Analysis</u>. All statistical data analyses were performed using SigmaStat and SigmaPlot software packages at α level of <0.05 (SPSS, Chicago, IL). Values are reported as mean+SEM.

RESULTS

Cellular phenotypes in the rat GP

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We have recorded from over 200 GP neurons. Consistent with the published reports, the predominant cellular phenotype encountered in our preparation (over 70%) possessed two cardinal electrophysiological properties both of which were recorded at the beginning of the experiments. The first property was a sag in membrane potential during a hyperpolarizing current injection in current clamp that corresponds to a time- and voltage-dependent inward current Ih. The second property was the presence of anodal breaks after a hyperpolarizing step, suggesting the presence of a low threshold-activated Ca²⁺ current It (Nambu and Llinas, 1994; Stanford and Cooper, 1999; Cooper and Stanford, 2000). These neurons were also characterized by a high input resistance $(712+150M\Omega)$ and spontaneous activity at rest (Fig. 1B). This cellular phenotype closely corresponds to type II or type A GP neurons previously described by Nambu & Llinas (1994), Cooper & Stanford (2000), and Shindou et al., (2001). Type II neurons are also thought to be the predominant cellular subtype encountered in the rat GP during in vivo recordings (Hassani et al., 1996). In the present study, we termed this neuronal subgroup type II GP neurons (Fig. 1B). We have also encountered a much less frequent cellular phenotype (less than 10%) that was characterized by the absence of I_h and I_t and presence of a ramp-like depolarization during a depolarizing current injection. These neurons were always quiescent at rest and possessed lower input resistance (405+20 M Ω). We termed these neurons type I GP neurons (Fig. 1A). This cellular subgroup corresponded to type C GP neurons previously described by Cooper and Stanford (2000).

In an attempt to correlate the morphology and relative position of GP neurons with their electrophysiological profiles, recorded neurons were filled with biocytin. However, we failed to find any consistent or significant differences in morphology or location between type I and type II GP neurons (Fig. 1C,D). About 20% of recorded GP neurons that displayed mixed electrophysiological properties of type I and type II GP neurons were not included in our analysis. Similarly, purported GP interneurons that are characterized by smaller cell bodies were excluded from our study (Millhouse, 1986; Cooper and Stanford, 2000).

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Stimulation of Group I mGluRs depolarizes type I and type II GP neurons

Previous immunocytochemical studies demonstrated that mGluR1a is expressed in the rodent GP (Testa et al., 1998) and both mGluR1 and mGluR5 are postsynaptically expressed in the primate external GP (GPe) (Hanson and Smith, 1999), but the possibility that both group I mGluRs are co-expressed in individual GP neurons has not yet been tested. To address this issue, we performed a double-labeling immunofluorescence study at the confocal microscope level. This set of experiments revealed that virtually all neurons in the GP display immunolabeling for both mGluR1a and mGluR5. Both receptors were found in the cell body, dendrites, and neuropil (Fig. 2).

Consistent with these immunocytochemical data, the group I selective agonist, DHPG, depolarized type I (Fig. 3) and type II GP neurons (Fig. 4) in the presence of 0.5 μ M TTX. The amplitude of the DHPG-induced depolarization was concentrationdependent in type II GP neurons and reached its maximum at 17±1.2 mV (Fig. 4C). Type I GP neurons were encountered so rarely in our preparation that we could not examine the dose-response relationship in this subgroup of GP neurons. Activation of group II and

group III mGluRs with selective agonists LY354740 and LAP4, respectively, had no effect on the membrane potential of either type I or type II (Figs. 3A,B, 4A,B). In type II GP neurons, stimulation of group I mGluRs with DHPG resulted in a consistent decrease in input resistance (Fig. 4A), whereas activation of group I mGluRs in type I GP neurons resulted in mixed effects on input resistance (data not shown).

Activation of group I mGluRs has been shown to affect a variety of conductances in different systems throughout the CNS. For instance, in hippocampal area CA3, DHPG depolarizes neurons by inhibition of a leak potassium conductance (Guerineau et al., 1994) or by an increase in a non-specific cationic conductance (Guerineau et al., 1995). Reduction in input resistance after stimulation with DHPG in type II GP neurons is consistent with an increase in conductance downstream of group I activation. We, therefore, tested whether activation of group I mGluRs depolarized type II GP neurons via a similar mechanism. To do so, we examined the current-voltage relationship of the group I mGluR-mediated inward current induced by application of DHPG (30 μ M). Application of DHPG induced a change in slope of the whole-cell current-voltage relationship (Fig. 5A). Subtracting the pre-drug IV trace from the trace in the presence of DHPG reveals a "V-shape" I-V relationship, the DHPG-induced current reversing polarity at two potentials: -9.3 ± 7 and -83.6 ± 13 (n=4, Fig. 5 inset and dotted boxes 1 and 2, respectively). Such an I-V relationship could indeed be explained by a mixed effect of DHPG. Group I mGluR activation could cause both a decrease of potassium outward currents (Charpak et al., 1990, Guerineau et al., 1994), which theoretically reverse at -95 mV, and an increase of a non-specific cationic current (Crepel et al., 1994, Guerineau et al., 1995), which theoretically reverses at 0 mV. Since application of DHPG induced a

decrease in input resistance together with a 15.9±1.7 mV depolarization (Fig. 4A and B), the increase in the net transmembrane conductance appears to be dominated by a group I mGluR-mediated increase in a non-specific cationic conductance at depolarized potentials.

Pharmacology of the DHPG-induced depolarization in the GP

Co-expression of both mGluR1a and mGluR5 has been reported in the STN, the SNr, and the striatum, three nuclei of the BG circuitry (Tallaksen-Greene, 1998; Awad et al., 2000; Marino et al., 2001b). However, the DHPG-induced depolarization and the potentiation of NMDA receptor currents in the STN is mediated solely by mGluR5 whereas activation of mGluR1 only is responsible for depolarization and induction of a slow excitatory postsynaptic potential (sEPSP) in SNr neurons (Awad et al., 2000, Marino et al., 2001b). Since GP neurons also co-express both group I mGluR sub-types (Fig. 2), we tested whether mGluR1 or mGluR5 mediated DHPG-induced depolarization. In type I GP neurons, 10 min-long pre-treatment with 100 µM LY367385, an mGluR1-selective antagonist, blocked the effect of DHPG on the membrane potential (p=0.011, one factor ANOVA, Tukey's pairwise comparison test, Fig. 3C,D). Pre-treatment with 10 µM MPEP, an mGluR5-selective antagonist, led to a small reduction in the amplitude of the DHPG-induced depolarization. This effect, however, was not statistically significant (Fig. 3C,D).

In type II GP neurons, the DHPG-induced depolarization was found to be mediated solely by mGluR1 (Fig. 4D, E), as pre-treatment with the mGluR1 selective antagonist LY363785 completely eliminated the response to DHPG. Interestingly, when

a type II GP neuron was exposed to MPEP prior to the application of the agonist, the response to DHPG was significantly potentiated (Fig. 4D,E; p=0.016, one factor ANOVA, Tukey's pairwise comparison test). Pre-treatment with MPEP did not alter the input resistance of these neurons (data not shown). Blockade of mGluR5 with MPEP also induced oscillations in the membrane potential during application of DHPG (Figure 4D, n=8). These oscillations were never observed when DHPG was applied alone. The mechanism that underlies these oscillations remains to be established.

Blockade of mGluR5 eliminates desensitization of mGluR1 in type II GP neurons

In the next series of experiments, we explored the mechanism(s) that underlies the potentiation of the mGluR1-mediated depolarization by mGluR5 blockade. We postulated that mGluR5 was involved in regulating the desensitization of mGluR1 and designed a series of experiments to test this hypothesis. We applied DHPG locally to the cell body of type II GP neurons for 20 seconds every 2 minutes. The mGluR1-mediated depolarization desensitized almost completely upon the second or third application of the agonist (Fig. 6A). In control experiments, the second application of DHPG elicited a depolarization that was 28.9±14.9% of the first response (Fig. 6B, control). However, if type II neurons were pre-treated with MPEP for 10 min prior to the first application of DHPG, the desensitization of mGluR1-mediated depolarization was blocked (Fig. 6A, bottom trace). In presence of MPEP, the second application of DHPG elicited a depolarization that was 80.08+18.0% in amplitude of the first response (Fig. 6B). There was no significant difference in the magnitude of the response between the first and second application of DHPG in the presence of MPEP (p=0.135, two factors repeated

measures ANOVA, Tukey's pairwise comparison test), which suggests that blockade of mGluR5 is sufficient to prevent the desensitization of the mGluR1-mediated depolarization.

PKC modulates the DHPG-induced activation of mGluR1 in type II GP neurons

We then investigated the mechanism(s) by which mGluR5 may regulate the desensitization of mGluR1. Group I mGluRs are known to activate and be regulated by PKC which has been shown to directly phosphorylate these receptors and diminish their coupling efficiency to G-proteins (Kawabata et al., 1996; Kawabata et al., 1998; Alagarsamy et al., 2001, review; De Blasi, 2001, review). We, therefore, postulated that mGluR5 might regulate mGluR1 by activation of PKC. To test this hypothesis, we included 1 µM bisendolylmaleimide (Bis), a broad-spectrum PKC blocker, in the intracellular solution and allowed it to diffuse into the cell for 10 min prior to the addition of DHPG. Consistent with our hypothesis, a 10 min pre-incubation with Bis potentiated the DHPG-induced depolarization in type II neurons (Fig. 7B; p=0.009, one factor ANOVA, Tukey's pairwise comparison test). The effect of Bis on the response to DHPG can be also replicated with another PKC blocker, chelerythrine chloride (Chel) (Jarvis et. al., 1994). Our data showed that a 10 min-long pre-incubation with 1 µM Chel resulted in a much stronger potentiation of the DHPG-induced depolarization than with Bis (Fig 7B, p<0.001, one factor ANOVA, Tukey's pairwise comparison test). We chose Bis for all subsequent experiments because we found that a 10-min long pre-incubation with Chel was often toxic to the cells and made recordings difficult.
Thus, blockade of PKC activity with either Bis or Chel results in a potentiation of the DHPG-induced depolarization in type II GP neurons. Conversely, a 10 min preincubation with PMA, a general PKC activator, which was also included in the intracellular solution, significantly reduced the response to the stimulation with DHPG in these neurons (Fig. 7B; p=0.023, one factor ANOVA, Tukey's pairwise comparison test). We used PMA at 10 and 100 nM and found no significant difference. We, therefore, pooled data obtained with the two concentrations in Fig. 7B. To assert the specificity of this drug, we evaluated the effect of $4-\alpha$ -PMA, an inactive analog of PMA, on the DHPG-induced depolarization and found that 100 nM, $4-\alpha$ -PMA did not significantly alter the DHPG-activated response in type II GP neurons (Fig 7B; p=0.697, one factor ANOVA, Tukey's pairwise comparison test).

Next, we assessed whether the effects of mGluR5 blockade with MPEP and the elimination of PKC activity with Bis on the DHPG-induced depolarization were additive. These experiments revealed that a 10 min-long incubation with MPEP and Bis did not alter the response to DHPG compared to incubation with MPEP or Bis alone (Fig.7D; p=0.821 and p=0.997 respectively, one factor ANOVA, Tukey's pairwise comparison test). In the next set of experiments we tested whether PMA could still exert its effect in presence of MPEP. Our findings, indeed, showed that a 10 min-long pre-incubation with 100 nM PMA still reduced the DHPG-induced depolarization in presence of MPEP when compared to MPEP alone (Fig. 7D, p=0.017, one factor ANOVA, Tukey's pairwise comparison test).

Blockade of PKC prevents the desensitization of mGluR1 in type II GP neurons

Since inhibition of PKC mimicked the effect of blocking mGluR5 on the DHPGinduced depolarization, we sought to investigate whether PKC regulates the desensitization of mGluR1 in the same manner as mGluR5. Indeed, a 10 min diffusion of Bis into the cell completely prevented the desensitization of the mGluR1-mediated depolarization (Fig. 8A). In presence of Bis, the second application of DHPG elicited a depolarization that was 91.95±14.75% of the first response. There was no significant difference between the magnitude of the response after the first and second application of DHPG when PKC activity was blocked (Fig. 8B; p=0.806, two factors repeated measures ANOVA, Tukey's pairwise comparison test). Taken together, these data suggest that both PKC and mGluR5 activity are required for agonist-induced desensitization of mGluR1 in type II GP neurons.

DISCUSSION

Data presented in this study reveal a novel type of functional interaction between mGluR1 and mGluR5 in the CNS. Our findings demonstrate that mGluR5 can regulate mGluR1 signaling by receptor desensitization. This mode of interaction constitutes an interesting form of heterologous desensitization in which there is an absolute requirement for activation of two receptors for the same neurotransmitter to achieve normal desensitization of the agonist-induced response. While heterologous desensitization is commonly observed in many receptor families, this most often occurs in a context in which a receptor is also capable of homologous desensitization. Also, heterologous desensitization often provides a mechanism for cross talk between two neurotransmitter systems. The heterologous desensitization described here is somewhat unique since the target (mGluR1) does not undergo desensitization without co-activation of another receptor that is responsive to the same neurotransmitter (mGluR5).

Functional Interactions Between mGluR1 and mGluR5 in GP Neurons

Three sets of data presented in this study suggest that the mGluR1/mGluR5 interaction is likely to be mediated by protein kinase C (PKC). First, the desensitizing effects of mGluR5 activation on mGluR1 responses can be mimicked by PKC activation (Fig.8): Second, PKC blockade potentiates the mGluR1 response to the agonist in a manner similar to that for the mGluR5 antagonist (Fig.7B). Third, the effects of blocking both mGluR5 and PKC on mGluR1 responses are not additive (Fig. 7D). However, the exact mechanism(s) by which PKC elicits its effects on mGluR1 responses remain to be established. Previous data suggest that two possibilities should be

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considered, either a direct phosphorylation of the receptor or desensitization of the effector systems downstream of mGluR1 activation (for review, see Ferguson, 2001 or Choe and Wang, 2002). The finding that both the mGluR5 antagonist and PKC inhibitors virtually eliminate mGluR1 desensitization suggests that the desensitization of the agonist-induced response is solely mediated by PKC and that mGluR5 likely acts through this mechanism. However, the possibility that additional PKC-independent mechanisms are involved in the mGluR5 regulation of mGluR1 desensitization cannot be ruled out (Dale et al., 2000; Sallese et al., 2000; Ferguson, 2001; Choe and Wang, 2002).

Metabotropic GluR5 can also undergo desensitization in a PKC-dependent manner (Gereau and Heinemann, 1998; Alagarsamy et al, 1999). Moreover, the desensitization state of mGluR5 may in fact alter its G protein coupling and result in the stimulation of different signaling systems (Rodriguez-Moreno et al, 1998; Herrero et al, 1998; Bruno et al., 2001). If this is true for our system, the desensitization of mGluR5 with manipulating PKC activity may also result in the potentiation of the DHPG-induced response. Then one would expect that PMA, a PKC activator, would not have an effect when mGluR5 is blocked. Our data, however, suggest that such is not the case here. A 10-min long incubation with PMA still reduced the amplitude of DHPG induceddepolarization in presence of MPEP. (Fig 7D). Therefore, PKC modulates the desensitization state of mGluR1 or the signaling system down-stream of mGluR1 activation.

Regardless of the exact mechanism by which mGluR5 desensitizes mGluR1, these data are intriguing in that they reveal that mGluR5 controls signaling of mGluR1 through receptor desensitization. Homologous desensitization of mGluR1 constitutes

only a minor portion of the mechanism regulating the signaling of this receptor (Figure 6). This implies that mGluR1 may not be fully capable of activating PKC or a critical PKC isoform in type II GP neurons. Alternatively, mGluR1 and mGluR5 may activate different pools of PKC such that PKC activated by mGluR1 may not have access to mGluR1 as a substrate. It is conceivable that the relevant PKC isoform exists in a signaling complex that is organized such that the enzyme is preferentially activated by mGluR5 but not mGluR1.

Differential Roles of mGluR1 and mGluR5 in the CNS

It is generally believed that mGluR1 and mGluR5 can couple to and activate the same second messenger cascades. However, the use of subtype-specific antagonists revealed that the two group I mGluRs possess unique functions that vary between different brain structures. For instance, mGluR1 activation mediates the DHPG-induced depolarization and intracellular Ca²⁺ release whereas mGluR5 modulates the Ca²⁺ - activated K⁺ current I_{AHP} in pyramidal cells of the CA1 region of the rat hippocampus (Mannaioni et al., 2001). On the other hand, activation of both mGluR1 and mGluR5 is required to increase intracellular Ca²⁺ release in SNr neurons, while activation of mGluR1 only leads to membrane depolarization (Marino, 2001b). In contrast, mGluR5, but not mGluR1, activation results in the depolarization of STN neurons despite a high level of neuronal co-expression of both receptor subtypes (Awad et al., 2000). Our data provide further evidence for different functions of mGluR1 and mGluR5 in the rat GP. It is unlikely that the mGluR5-mediated effects on mGluR1 response described in the present study occur in all neurons that co-express the two group I mGluR subtypes. For

instance, such interactions were not seen in SNr, STN or hippocampal neurons (Awad et al., 2000; Mannaioni et al., 2001; Marino et al., 2001b). Even in the GP itself, the mGluR5-mediated desensitization of mGluR1 activity was found in type II, but not type I neurons. Together, these observations provide strong evidence for specific, differential and complementary functions of the two group I mGluR subtypes in the CNS. The specificity of the mGluR1 and mGluR5 functions may be determined by the unique composition of the synaptic signaling complexes or scaffolds that associate with these receptors (for review, see Thomas, 2002).

mGluR5 Antagonists and Parkinson's Disease

The observation that blockade of mGluR5 potentiates mGluR1-mediated depolarization of most GP neurons is of interest in the search for new therapeutic targets for the treatment of Parkinson's Disease (PD). PD is a debilitating motor disorder characterized by akinesia, bradykinesia, and tremor. Hyperactivity of the STN has long been associated with some of the hallmark symptoms of the disease (for review, see DeLong, 1990). In the STN, mGluR5 mediates excitatory effects (Awad et al., 2000). Thus, blockade of mGluR5 activity in the STN can be beneficial in treating PD pathophysiology. Indeed, there are reports demonstrating that systemic administration of MPEP ameliorates parkinsonian-like symptoms in rodent models of the disease (Ossowska et al., 2001; Spooren WP, 2001, review; Breysse et al., 2002). Our findings provide support for another mechanism by which silencing mGluR5 in the GP may also be beneficial in PD. Based on our observation that MPEP potentiates the mGluR1mediated depolarization of GP neurons combined with the fact that the GP sends a

massive inhibitory projection to the STN, one can speculate that MPEP exerts its antiparkinsonian effects by facilitating the mGluR1-mediated increased activity of the pallidosubthalamic pathway. This would attenuate the hyperactive glutamatergic subthalamofugal projection to basal ganglia output structures, thereby, facilitating transmission through the basal ganglia-thalamocortical loops. It is noteworthy that group II and group III mGluRs also represent additional potential targets for future therapeutic strategies in Parkinson's disease (for review, see Conn et al., 2000; Rouse et al., 2000; or Valenti et al., 2002).

Concluding Remarks

In conclusion, data obtained over the past few years have clearly shown that the three groups of mGluRs are widely distributed throughout the basal ganglia where they play various functions at pre- and post-synaptic levels to regulate GABAergic and glutamatergic transmission (Conn et al., 2000; Rouse et al., 2000; Smith et al., 2001; Valenti et al., 2002). Our findings suggest these receptors may be important in regulating neurotransmission in the basal ganglia and pave the way for the development of novel therapeutic strategies in Parkinson's disease.

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FIGURE LEGENDS

Figure 1. Electrophysiological and morphological profiles of type I and type II GP neurons. (**A**) Type I GP neurons are characterized by the presence of a ramp-like depolarization during a depolarizing current injection, lack of time and voltage-dependent current I_h, and low input resistance. (**B**) Type II GP neurons are characterized by lack of ramp depolarization during a depolarizing current injection, presence of time and voltage-dependent current I_h, activity at rest, presence of rebound depolarization after a hyperpolarizing step, and high input resistance. (**C**) No consistent differences in cellular morphology are observed between type I (**i**) and type II (**i**) GP neurons. (**D**) No consistent differences in the position of cell body or dendritic arborization were observed between type I (**i**) and type II (**ii**) GP neurons. Str- striatum, GP – globus pallidus. Scale bars-**C**: 20 µm, **D**: 100 µm.

Figure 2. MGluR1a and mGluR5 are co-localized in rat GP neurons. (**A**) Low power micrograph of mGluR1a immunoreactivity in the GP. (**B**) High power micrograph of the same field showing neuronal cell bodies immunoreactive for both mGluR1a (**i**) and mGluR5 (**ii**). <u>Abbreviations</u>: Str- striatum, GP – globus pallidus. <u>Scale bars</u>: **A**: 200 μm. **B**: 15 μm.

Figure 3. Activation of mGluR1 depolarizes type I GP neurons. (A) Type I GP neurons are depolarized by 30 μ M DHPG, a group I-selective agonist, whereas group II and III selective agonists LY354740 and LAP4, respectively, do not change the membrane potential in these cells. (B) Mean + SEM of data for type I GP neurons, number of

cells/condition is given above each bar in parentheses. The asterisk (*) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure. (C) The DHPG-induced depolarization is predominantly mediated by mGluR1 in type I GP neurons. (D) Mean \pm SEM of data for type I GP neurons, number of cells/condition is given above each bar in parentheses. The asterisk (*) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure. TTX (0.5 μ M) was bath applied for at least 5 min before the beginning of all experiments. All antagonists were bath-applied for 10 min prior to exposure to DHPG.

Figure 4. Pharmacology of group I-mediated depolarization in type II GP neurons. (A). Activation of the group I mGluRs with 30 μ M DHPG causes a depolarization and reduces the input resistance in type II GP neurons while group II and III selective agonists LY354740 and LAP4, respectively, do not change the membrane potential or the input resistance in these cells. (B) Mean <u>+</u> SEM of data for type II GP neurons, number of cells/condition is given above each bar in parentheses. The asterisk (^{*}) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure. (C) Dose-response relationship for DHPG-induced depolarization in type II GP neurons. (D) MGluR1 solely mediates DHPG-induced depolarization in type II GP neurons. Pre-incubation with the mGluR1-selective antagonist, LY363785, abolishes the DHPG-induced depolarization, whereas, pre-incubation with MPEP, an mGluR5-selective blocker, potentiates the response to DHPG. (E) Mean + SEM for type II GP neurons, number of cells/condition is

given above each bar in parentheses. The asterisk (^{*}) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure.

Figure 5. DHPG-induced current reverses polarity at two membrane potentials in type II GP neurons. The group I-mediated depolarization observed in type II GP neurons is associated with an increase in membrane conductance (Fig. 4A). This increase in membrane conductance is evident in the whole cell current-voltage relation shown in this figure. The inset shows the subtraction of the currents that reveals a "V-shape" relationship with two distinct potentials, at which the current polarity is reversed (see also the dotted boxes 1 and 2). Axis titles apply in the inset. This figure is representative of results observed in 4 cells.

Figure 6. Blockade of mGluR5 reverses the desensitization of the mGluR1-mediated depolarization in type II GP neurons. MGluR1-mediated depolarization desensitizes upon repeated application of 100 μ M DHPG (**A**, *top trace*.). Pre-treatment with 10 μ M MPEP, an mGluR5-selective antagonist, for 10 min prior to the first application of 100 μ M DHPG reverses the desensitization of the mGluR1-mediated depolarization (**A**, *bottom trace*.). (**B**) Mean ± SEM of data for 5 type II GP neurons/condition. The asterisk (*) denotes statistical significance and difference from the first response to DHPG as determined by two factor repeated measures ANOVA (α >0.05) and Tukey's pairwise comparison procedure.

Figure 7. PKC regulates mGluR1 response to DHPG in type II GP neurons. (**A,B**) Blockade of PKC with 1 μ M Bis or 1 μ M Chel potentiates mGluR1-mediated depolarization, while activation of PKC with 10-100 nM PMA reduces it. Bis , Chel, PMA, or 4- α -PMA were included in the intracellular solution and allowed to diffuse into the cell for 10 min prior to the bath application of DHPG. (**B**) Mean <u>+</u>SEM of data for type II GP neurons, number of cells/condition is given above each bar in parentheses. The asterisk (^{*}) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure. (**C,D**) The effects of Bis and MPEP are not additive. PMA still has an effect on the mGluR1-mediated response to DHPG in presence of MPEP. (**D**) Mean <u>+</u>SEM of data for type II GP neurons, number of cells/condition is given above each bar in parentheses. The asterisk (^{*}) denotes statistical significance and difference compared to DHPG as data for type II GP neurons, number of cells/condition is given above each bar in parentheses. The asterisk (^{*}) denotes statistical significance and difference compared to DHPG as determined by one factor ANOVA (α >0.05) and Tukey's pairwise comparison procedure.

<u>Figure 8.</u> PKC regulates the desensitization of mGluR1 in type II GP neurons. Metabotropic GluR1-mediated depolarization desensitizes upon repeated activation with 100 μ M DHPG (**A**, *top trace*.). In presence of 1 μ M Bis, a PKC blocker, which was included in the intracellular solution and allowed to diffuse into the cell for 10 min prior to the first application of 100 μ M DHPG, the desensitization of the mGluR1-mediated depolarization is reversed (**A**, *bottom trace*.). (**B**) Mean ± SEM of data for 4 type II GP neurons/condition. The asterisk (*) denotes statistical significance and difference from

the first response to DHPG as determined by two factors repeated measures ANOVA (α >0.05) and Tukey's pairwise comparison procedure.

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REGULATION OF NEURONS OF THE SUBTHALAMIC NUCLEUS BY METABOTROPIC GLUTAMATE RECEPTORS. <u>H. Awad*, P.J. Conn.</u> Department of Pharmacology, Emory University School of Medicine and Graduate Program in Molecular Therapeutics and Toxicology, Graduate Division of Biological and Biomedical Sciences, Atlanta, GA 30322.

Hyperactivity of neurons of the subthalamic nucleus (STN) is involved in the pathophysiology of various movement disorders, including Parkinson's disease. Agents that reduce excitation of the STN could have a therapeutic effect in the treatment of these disorders. Here we investigate the role of metabotropic glutamate receptors (mGluRs) in the regulation of STN neuron activity.

Electrophysiological recordings were made from STN neurons in horizontal rat brain slices. The group I selective mGluR agonist dihydroxyphenylglycine (DHPG 100µM), but not the group II agonist (+)-2-aminobicyclo[3.1.0]hexane-2,6dicarboxylate (LY354740 100nM) or the group III agonist L-2-amino-4phosphonobutyrate (L-AP4 1mM), caused a direct depolarization of STN neurons This group I-mediated depolarization was accompanied by a $(17.1 \pm 1.1 \text{mV})$. marked increase in cell firing and an increase in input resistance. The I-V curve shows a reversal potential of -80mV, consistent with a group I-mediated inhibition of a potassium channel. DHPG-mediated depolarization is significantly attenuated by the mGluR5 selective antagonist methylphenylethynylpyridine (MPEP 10µM; 4.2±0.3mV) but not the mGluR1 selective antagonist 7-hydroxyiminocyclopropan-[b]chromen-la-carboxylic acid ethyl ester (CPCCOEt 100µM; 18.8±3mV). The mGluR5 selective agonist, (S)(+)-2-(3'-carboxybicyclo[1.1.1]-pentyl)-glycine (CBPG 100µM) mimicks the depolarizing effect of DHPG and is also blocked by MPEP but not CPCCOEt. These data suggest that mGluR5 mediates depolarization of STN neurons. We are currently investigating the effect of mGluR activation on ionotropic glutamate and GABA receptors in STN neurons and the role of mGluRs in modulation of transmission at excitatory and inhibitory synapses onto STN neurons. (Supported by grants from NIH NINDS, and the U.S. Army).

PHYSIOLOGICAL ROLES OF PRESYNAPTICALLY LOCALIZED TYPE 2,3 AND 7 METABOTROPIC GLUTAMATE RECEPTORS IN RAT BASAL GANGLIA <u>S. Risso Bradley^{*,1}, M.J. Marino¹, M. Wittmann², S.</u> <u>Rouse¹, A.I. Levey³, and P. J. Conn¹</u>, Depts of Pharmacology¹ and Neurology³, Emory Univ. Sch. of Med. Atlanta, GA 30322, and Tierphysiologie², Univ. Tuebingen, D-72076 Tuebingen, Germany.

Metabotropic glutamate receptors (mGluRs) play a significant role in regulating basal ganglia (BG) function. BG are a set of interconnected subcortical nuclei that play a major role in the control of movement and pathophysiology of movement disorders. The input nucleus of the BG is the striatum, which receives innervation from the cortex. The primary output nuclei are the substantia nigra pars reticulata (SNpr) and the entopeduncular nucleus, which send inhibitory projections to the thalamus. The SNpr receives glutamatergic excitation from the subthalamic nucleus (STN). Hypokinetic movement disorders, such as Parkinson's disease (PD), in part result from an increased excitation of SNpr. Our immunocytochemistry studies at the electron microscopic level reveal that mGluR2/3 and mGluR7 are presynaptically localized in the SNpr on glutamatergic synapses. Therefore, we investigated the physiological roles of mGluR2/3 and 7 in rat slices of SNpr using the patch clamp technique in whole cell configuration. Our experiments suggest that activation of mGluR2/3 and mGluR7 inhibits excitatory transmission at the STN-SNpr synapse by a presynaptic mechanism. These data support the hypothesis that mGluR2/3 and mGluR7 may act as presynaptic receptors in the SNpr, where they play an important role in regulating glutamate release from STN-SNpr terminals. This provides a strong basis for development of novel therapeutic agents that target specific mGluR subtypes and could be used for treatment of PD and other disorders involving pathological changes in BG function. Supported by grants from NIH NINDS and U.S. Army.

GABAERGIC INHIBITION OF RAT SUBSTANTIA NIGRA PARS RETICULATA PROJECTION NEURONS IS MODULATED BY METABOTROPIC GLUTAMATE RECEPTORS. <u>M. Wittmann[†], M.J. Marino, S.</u> <u>Risso Bradley, and P.J. Conn^{*}</u>. Depts of Pharmacology, Emory University, Atlanta, GA 30322 and [†] Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

The predominant inputs to the substantia nigra pars reticulata (SNr) are GABAergic projections from the striatum and the globus pallidus. Since over excitation of SNr output neurons is believed to play an important role in the pathophysiology of Parkinson's disease, the modulation of these GABAergic inputs may provide a crucial target for drug development.

Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG. The predominant postsynaptic mGluRs are a group I mGluRs, while group II and III mGluRs are often localized presynaptically. This would indicate that group II and III mGluRs are likely to play a role in presynaptic modulation of transmitter release. Here we examine the role of mGluRs in regulation of inhibitory synaptic transmission in the SNr. Application of the group I-selective agonist DHPG (100 µM) and the group III-selective agonist L-AP4 (500 µM) inhibit evoked IPSCs obtained by whole cell patch clamp recording from SNr GABAergic projection neurons. The group IIselective mGluR agonist LY354740 (100 nM) had no effect on the amplitude of the IPSCs. Interestingly, in other studies we have found that activation of group I mGluRs directly depolarize SNr GABAergic neurons. These findings suggest that activation of group I mGluRs can excite GABAergic projection neurons both by direct stimulation, and by disinhibition. Thus, group I mGluR antagonists could provide novel therapeutic targets for treatment of Parkinson's disease. Current work is aimed at more fully characterizing the pharmacology of this effect, and at determining the pre- or post-synaptic locus of this synaptic inhibition.[Supported by NIH NINDS and the U.S. Army]

APPENDIX XVIII

740.15

GLUTAMATE RECEPTORS MODULATE EXCITATORY AND METABOTROPIC INHIBITORY TRANSMISSION IN SUBSTANTIA NIGRA PARS RETICULATA. M.

Wittmann, M.J. Marino, S.T. Rouse^{*}, P.J. Conn. Dept. of Pharmacology, Emory University,

The Substantia nigra pars reticulata (SNr) is one of the main output structures of the basal ganglia (BG), a highly connected set of subcortical nuclei. Cortical information is processed through the BG to the SNr via two major pathways. The direct pathway which provides a GABAergic inhibition of the SNr projection neurons and the indirect pathway projecting through globus pallidus (GP) and subthalamic nucleus (STN) and resulting in a glutamatergic excitation of the output neurons. A delicate balance between these two inputs is believed to be crucial for initiation of movement, and imbalance in this system underlies the pathophysiology of movement disorders. Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of BG function. We have previously shown that presynaptically localized group-II mGluRs inhibit excitatory transmission at the STN-SNr synapse. We have now investigated the role of other mGluR subtypes in the modulation of inhibitory and excitatory transmission in SNr We have found that group-III mGluRs also inhibit EPSCs at the STN-SNr synapse and that group-I and group-III mGluRs decrease IPSCs in the SNr. Miniature IPSC studies and paired pulse studies indicate that these inhibitory effects on EPSCs and IPSCs in SNr neurons are mediated by presynaptic mechanisms. We are currently further investigating the modulation of presynaptic mGluR effects at the STN-SNr synapse. It is known that cyclic AMP analogs and forskolin, an activator of adenylyl cyclase, can inhibit the function of presynaptic group-II mGluRs. Preliminary data reveal that forskolin (50µM) inhibits the presynaptic effect of the group-II specific agonist LY354740 (100nM) at the STN-SNr synapse. Current work is aimed at further investigating this effect and its implications on BG function. Supported by: NIH NINDS and the U.S. Army.

DIRECT EXCITATION OF GABAERGIC PROJECTION NEURONS OF THE RAT SUBSTANTIA NIGRA PARS RETICULATA BY ACTIVATION OF THE MGLUR1 METABOTROPIC GLUTAMATE RECEPTOR. <u>M.J. Marino*</u>, <u>S. Risso Bradley, M. Wittmann[†], and P.J. Conn.</u> Dept. of Pharmacology, Emory University, Atlanta, GA 30322 and, [†] Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG. The predominant postsynaptic mGluRs are a group I mGluRs, while group II and III mGluRs are often localized presynaptically. This would indicate that group I receptors are localized in a manner consistent with direct modulation of the excitability of projection neurons. However, little is known about the physiological roles mGluRs play in regulating the function of BG structures. Here we demonstrate that activation of mGluRs by the group 1 mGluR selective agonist DHPG (30 µM - 1 mM) produces a direct depolarization of GABAergic projection neurons in the substantia nigra pars reticulata (SNr) along with a concomitant increase in input resistance. This effect is not mimicked by application of the group II - selective mGluR agonist LY354740 (100 nM) or by the group III-selective mGluR agonist L-AP4 (500 μ M). Furthermore, the DHPG-induced depolarization is blocked by the mGluR1 subtype selective antagonist CPPCCOEt (100 μ M), but is not blocked by the mGluR5 subtype selective antagonist MPEP (10 μ M), and is not mimicked by the mGluR5 subtype selective agonist CBPG (100 µM). This provides strong evidence that the direct excitation of SNr projection neurons is mediated by mGluR1. Since the over excitation of SNr GABAergic neurons is believed to play a major role in the pathophysiology of Parkinson's disease, mGluR1 may provide an important target for new therapeutic agents that could be useful for treatment of this disorder. [Supported by NIH NINDS and the U.S. Army]

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APPENDIX XX

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PHYSIOLOGICAL ROLES OF MULTIPLE METABOTROPIC GLUTAMATE RECEPTOR SUBTYPES IN RAT BASAL GANGLIA

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U.S.A.

The primary pathophysiological change giving rise to the symptoms of Parkinson's disease (PD) is loss of substantia nigra dopaminergic neurons that are involved in modulating function of the striatum and other basal ganglia structures. Unfortunately, traditional therapies for treatment of PD based on dopamine replacement strategies eventually fail in most patients. Because of this, a great deal of effort has been focused on developing a detailed understanding of the circuitry and function of the basal ganglia in hopes of developing novel therapeutic approaches for restoring normal basal ganglia function in patients suffering from PD. We have performed a series of studies of the distribution and function of mGluR subtypes in the basal ganglia that suggest that members of this receptor family could serve as targets for novel therapeutic agents that would be effective in treatment of PD. For instance, we found that two group III mGluRs (mGluR4 and mGluR7) are localized on presynaptic terminals of striatal neurons in the globus pallidus where they could reduce GABA release. Furthermore, activation of group I mGluRs results in a depolarization and increased cell firing of neurons in the subthalamic nucleus (STN) and projection neurons of the substantia nigra pars reticulata (SNpr). Interestingly, studies with subtype-selective adonists and antagonists suggest that this effect is mediated by

mGluR1 in SNpr projection neurons and mGluR5 in STN neurons. Finally, we found that activation of group II mGluRs results in inhibition of glutamate release from STN terminals in the SNpr. Furthermore, selective agonists of group II mGluRs inhibit haloperidol-induced catalepsy in rats, suggesting an antiparkinsonian effect of these compounds. The rich distribution and diverse physiological roles of mGluRs in basal ganglia raises the possibility that these receptors may provide targets for novel therapeutic agents that could be used for treatment of PD and related disorders.

Oral presentation preferred

METABOTROPIC GLUTAMATE RECEPTORS MODULATE EXCITATORY TRANSMIS SION IN THE RAT SUBTHALAMIC NUCLEUS. H. Awad^{*}, P.J. Conn. Department of Phan

macology, Emory University School of Medicine, Graduate Program in Molecular and Systems Pharmacology, Atlanta, GA

Overactivity in neurons of the subthalamic nucleus (STN) has been shown to be a major pathophysiological change that occurs in Parkinson's disease. Agents that reduce the activity of the STN may have beneficial therapeutic effects in Parkinson's disease. The primary afferents to the STN arise from the cortex, globus pallidus, thalamus and pedunculopontine nucleus (PPN). Here we examine the role of metabotropic glutamate receptors (mGluRs) in modulation of excitatory and inhibitory synaptic transmission in the STN. Electrophysiological recordings were made from STN neurons in parasaggital rat brain slices. Stimulating electrodes were placed in the internal capsule (IC) for stimulating descending afferents and in the cerebral peduncle (CP) for stimulating ascende ing afferents. Excitatory post-synaptic currents (EPSCs) were elicited in the STN in the presence of 10µM Bicuculline, and inhibitory post-synaptic currents (IPSCs) were elicited in the presence of 20µM CNQX and 20µM D-AP5. Under IC stimulation, the group I selective mGluR agonist DHPG (100µM) caused a 34.3±3.3% reduction of EPSCs, the group II agonist LY354740 (100nM) caused a 43.5±6.8% reduction in EPSCs, and the group III agonist L-AP4 (1mM) caused a 80.9±6.7% reduction in EPSCs. On the other hand, only group I and III mGluR activation, but not group II, caused a reduction in EPSCs elicited by CP stimulation of ascending fibers into the STN. mGluR activation had no effect on IPSC amplitude in the STN. These data suggest differ ential effects of mGluR activation on synaptic transmission arising from different afferents onto STN neurons. We are currently further investigating this mGluR inhibition of EPSCs to determine whether this effect is due to a presynaptic or postsynaptic modulation. Supported by: grants from NIH NINDS, and the U.S. Army.

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DISTINCT FUNCTIONS OF GROUP I METABOTROPIC GLUTAMATE RECEPTORS 1 AND 5 IN THE RAT GLOBUS PALLIDUS

Poisik, O.¹, Mannaioni, G.², Traynelis, S.², Smith, Y.¹³, & Conn, P.J.⁴. ¹Yerkes Regional Primate Center, and Departments of ²Pharmacology and ³Neurology, Emory Univ. Sch. of Med., Atlanta, GA, 30322. Merck Research Laboratories, West Point, PA 19486.

Group I metabotropic glutamate receptors (mGluRs) 1 and 5 co-localize to the same neurons in the rat globus pallidus (GP). Since both receptors can couple to the same effector systems, the goal of this study was to investigate the role of mGluR1 and mGluR5 in type II GP neurons. Type Il cells represent the predominant neuronal phenotype in the rat GP. These are GABAergic projection neurons characterized by the presence of time and voltage-dependent inward rectification, spontaneous firing activity at rest, and a high input resistance of 813 ± 159 MQ. Using whole cell recording in brain slices of young (P14-18) rats, we report that activation of either mGluR1 or mGluR5 with the group I selective agonist (RS)-3,5 DHPG has different effects on the membrane potential of type II GP neurons. The DHPG-induced depolarization is entirely mediated by mGluR1 as pre-treatment with LY367385, a highly selective mGluR1 antagonist, completely eliminates the response. The mGluR1-mediated depolarization is accomplished via modulation of K⁺ and non-specific cationic conductances. We found that blockade of mGluR5 with the selective antagonist MPEP, potentiates DHPG-induced depolarization and induces oscillations in the membrane potential of type II GP neurons. Blockade of mGluR5 also changes the response of mGluR1 to repeated applications of DHPG. The mGluR1-mediated response dramatically desensitizes upon repeated activation of the receptor with the agonist. When the cell is pretreated with MPEP, the desensitization is blocked. We demonstrate that this functional role of mGhuR5 on the desensitization of the mOluR1-mediated response can be mimicked by manipulating the activity of protein kinase C.

These results suggest that mGluR1 and mGluR5 have functionally distinct roles in type II GP neurons, and that mGluR5 may regulate the desensitization of mGluR1 via activation of protein kinase C. Supported by NIH NINDS and the US Army.

FUNCTIONAL ROLES OF GROUP I METABOTROPIC GLUTAMATE RECEPTORS IN THE SUBSTANTIA NIGRA PARS RETICULATA. M.J. Marino^{*}, G.W. Hubert, Y. Smith,

P.J. Conn. Dept. of Pharmacology, and Neurology, Emory University, Atlanta, GA, USA Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG The predominant postsynaptic mGluRs are the group I mGluRs (mGluR1 and mGluR5), while group II and group III mGluRs are often localized presynaptically. This would indicate that group I receptors are localized in a manner consistent with direct modulation of the excitability of projection neurons. Since the over excitation of substantia nigra pars reticulata (SNr) GABAergic neurons is believed to play a major role in the pathophysiology of Parkinson's disease, group I mGluRs may provide an important target for new therapeutic agents that could be useful for treatment of this disorder. However, little is known about the physiological roles mGluRs play in regulating the function of BG structures Previously, we have shown that the group I mGluR agonist DHPG produces a direct depolarization of GABAergic projection neurons in the SNr. Pharmacological studies reveal that this DHPG-induced depolarization is mediated by mGluR1. This is somewhat surprising, as we have found that both mGluR1 and mGluR5 are localized postsynaptically in SNr projection neurons, and these receptors are known to couple to the same signal transduction systems. We now report that activation of group I mGluRs induces a potentiation of NMDA-receptor currents in SNr projection neurons. Preliminary results indicate that this effect is also mediated by mGluR1. Current studies are aimed at determining the physiological significance of this modulation, and at investigating the role mGluR5 receptor activation plays in these cells. Supported by: NIH NS98011, RR00165, and grants from The U.S. Army, and The National Parkinson's Foundation.

740.17 FUNCTIONAL ROLES OF GROUP I METABOTROPIC GLUTAMATE RECEPTORS IN TWO NEURONAL POPULATIONS IN RAT GLOBUS PALLIDUS. O. Maltseva, P.J. Conn

Department of Pharmacology, Emory Univ. Sch. of Med., Atlanta, GA Three types of neurons have been characterized in rat globus pallidus (GP) based on morphological and electrophysiological properties. Although all three types are GABAergic, they are thought to be functionally distinct. In this study, whole-cell patch clamp recording in brain slices was used to investigate the functional roles of group I metabotropic glutamate receptors (mGluRs) in the two predominant cell types in GP, type I and type II. Type II neurons were characterized by time and voltage-dependent inward rectification. These cells exhibited input resistance of 813 ± 159 M Ω . Rebound depolarization was observed in 40% of these cells. Type I GP neurons were characterized by lack of time and voltage-dependent inward rectification. These cells exhibited significantly lower input resistance of 405±20 MΩ. (RS)-3,5-DHPG (1-100 µM) was used to show the functional presence of group I mGluRs in these neuronal subtypes. CPCCOEt (100 μ M) and MPEP (10µM) were utilized to differentiate between mGluR1 and mGluR5, members of group I mGluRs. Type II GP neurons responded to 100 μ M DHPG with a robust depolarization of 16.6±8.7 mV from rest. Type I GP neurons, on the other hand, did not respond to 100 uM DHPG (5±3.8mV). Our results provide evidence that, in addition to their differences in membrane properties, these two GP neuronal populations may differ in their responses to activation of group I mGluRs. Supported by: NIH NIDS and the US Army...

APPENDIX XXV

METABOTROPIC GLUTAMATE RECEPTOR-MEDIATED REGULATION OF EXCITATORY TRANSMISSION IN THE RAT SUBSTANTIA NIGRA PARS RETICULATA

<u>M. J. Marino</u>, M. Wittmann, S.R. Bradley, and P. J. Conn, Emory University Dept. of Pharmacology, Atlanta, GA 30322 U.S.A.

Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG. The predominant postsynaptic mGluRs are a group I mGluRs, while group II and III mGluRs are often localized presynaptically. However, little is known about the physiological roles mGluRs play in regulating the function of BG structures. The group II mGluRs (mGluR2 and mGluR3) are expressed in neurons in the subthalamic nucleus (STN) and these receptors have been shown to regulate glutamate release in other brain regions. This led us to postulate that group II mGluRs are presynaptically localized on STN terminals in the substantia nigra pars reticulata (SNr) and that activation of these receptors would reduce excitatory synaptic responses. We have found that activation of presynaptically localized group II mGluRs inhibits excitatory transmission at the STN-SNr synapse. This suggests that a selective group II mGluR agonist could ameliorate the motor dysfunction associated with Parkinson's disease. Consistent with this, we find that the highly selective group II mGluR agonist LY354740 reverses catalepsy in an animal model of Parkinson's disease. In addition, the group I mGluRs (mGluR1 and mGluR5) are localized postsynaptically in SNr projection neurons. Here we demonstrate that activation of postsynaptically localized mGluR1 by exogenous agonists or synaptic glutamate produces a direct depolarization of GABAergic projection neurons in the SNr along with a concomitant increase in input resistance. In addition, activation of mGluR1 induces a potentiation of NMDA-receptor currents in SNr projection neurons. The fact that mGluR1 alone mediates these responses is somewhat surprising, as both mGluR1 and mGluR5 are known to couple to the same signal transduction systems. Since the over excitation of SNr GABAergic neurons is believed to play a major role in the pathophysiology of Parkinson's disease, mGluR1 may provide an important target for new therapeutic agents that could be useful for treatment of this disorder Supported by grants from NIH, US Army, and the National Parkinson's Foundation. Animals were anesthetized with chloral hydrate (700 mg/kg, IP) prior to decapitation.

DOPAMINE REGULATION OF METABOTROPIC GLUTAMATE RECEPTOR SIGNALING IN THE RAT SUBSTANTIA NIGRA PARS RETICULATA.

Michael J. Marino, Marion Wittmann, and P. Jeffrey Conn*

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The substantia nigra pars reticulata (SNr) is a primary output nucleus of the basal ganglia motor circuit Alterations in inhibitory and excitatory transmission in the basal ganglia output nuclei are known to play a major role ir a variety of movement disorders. We have found that metabotropic glutamate receptors (mGluRs) play important roles in modulating transmission and neuronal excitability in the SNr. For example, mGluR1 is postsynaptically localized in GABAergic SNr neurons and activation of this receptor with exogenous agonists or synaptic activation induces a direcdepolarization of SNr neurons. In addition, activation of presynaptic group I and group III mGluRs enhances SNr activity by decreasing inhibitory synaptic transmission. This combination of postsynaptic excitation, and disinhibition coulc exert a powerful excitatory influence on SNr output. Therefore, regulation of the mGluRs by alterations in the dopamine system could have broad implications for regulating basal ganglia function in both normal and pathophysiologica conditions. Interestingly, we found that in vivo treatment of rats with the dopamine receptor antagonist haloperido. produces an enhanced response of SNr neurons to group I mGluR activation that can be measured in midbrain slices 1 day after initiation of haloperidol treatment. This effect is mediated by expression of an mGluR5-mediated response that is not present in control rats. In addition, activation of cyclic AMP-dependent protein kinase inhibits the group III mGluR-mediated disinhibition of SNr neurons. It is possible that D1 dopamine receptors that are present on these nerve terminals could enhance synaptic inhibition in the SNr by a cAMP-mediated reduction in presynaptic mGluR function Supported by grants from NIH, US Army, and the National Parkinson's Foundation.

APPENDIX XXVI

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Program Number: 63.3 D a y / Time: Sunday, Nov. 3, 10:00 AM - 11:00 AM

SUBCELLULAR LOCALIZATION OF GROUP I I METABOTROPIC GLUTAMATE RECEPTORS

L.A.Iskhakova^{*}; M.Paquet; J.F.Pare; Y.Smith

Yerkes Primate Center, Div. Neurosci, Emory Univ, Atlanta, GA, USA

Pre- and post-synaptic metabotropic glutamate receptors (mGluRs) modulate excitatory synaptic transmission and participate in the induction of long-term depression (LTD) of glutamatergic synapses in the rat striatum. Group II mGluRs modulate presynaptically the release of glutamate in the striatum. In order to better understand the anatomical substrate that underlies striatal functions of group II mGluRs, we undertook an electron microscopy immunocytochemical analysis of the subcellular and subsynaptic localization of mGluR2/3 immunoreactivity (IR) in various striatal regions including the caudate nucleus, putamen, accumbens core and shell. Overall, the pattern of distribution of mGluR 2/3 IR was similar throughout the striatum. Immunostained small unmyelinated axons, preterminal axonal segments of glutamatergic boutons and glial cell processes were most commonly found in the immunoperoxidase-stained striatal tissue. A small population of dendrites of various sizes and spines also showed immunoreactivity, but labeled axon terminals were scarce. Pre-embedding immunogold technique revealed that 60%-70% of mGluR2/3 labeling was intracellular whereas 30%-40% of gold particles were bound to the plasma membrane. Of those membrane-bound particles, more than 95% were expressed at non-synaptic sites suggesting that mGluR 2/3 may be primarily activated by extrasynaptic diffusion of neurotransmitter. In conclusion, group II mGluRs are located to subserve pre- and post-synaptic functions in the monkey striatum, but only under physiological or pathological conditions that induce extrasynaptic spillover of glutamate. Supported by: NIH R01 NS37423, RR00165

Citation: L.A.Iskhakova, M.Paquet, J.F.Pare, Y.Smith. SUBCELLULAR LOCALIZATION OF GROUP II METABOTROPIC GLUTAMATE RECEPTORS IN THE MONKEY STRIATUM. Program No. 63.3. 2002 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2002. CD-ROM.

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