A Common Signaling Pathway for Striatal NMDA and Adenosine A_{2a} Receptors: Implications for the Treatment of Parkinson's Disease

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The striatum is the major input region of the basal ganglia, playing a pivotal role in the selection, initiation, and coordination of movement both physiologically and in pathophysiological situations such as Parkinson's disease. In the present study, we characterize interactions between NMDA receptors, adenosine receptors, and cAMP signaling within the striatum. Both NMDA (100 μ M) and the adenosine A_{2a} receptor agonist CPCA (3 μ M) increased cAMP levels ($218.9 \pm 19.9\%$ and $395.7 \pm 67.2\%$, respectively; cf. basal). The NMDA-induced increase in cAMP was completely blocked when slices were preincubated with either the NMDA receptor antagonist 7-chlorokynurenate or the adenosine A₂ receptor antagonist DMPX (100 μ M), suggesting that striatal NMDA receptors increase cAMP indirectly via stimulation of adenosine A_{2a} receptors. Thus, NMDA receptors and

adenosine A_{2a} receptors might share a common signaling pathway within the striatum. In striatal slices prepared from the 6-hydroxydopamine-lesioned rat model of Parkinson's disease, NMDA receptor-mediated increases in cAMP were greater on the lesioned side compared with the unlesioned side ($349.6 \pm 40.2\%$ compared with $200.9 \pm 21.9\%$ of basal levels, respectively). This finding substantiates previous evidence implicating overactivity of striatal NMDA receptors in parkinsonism and suggests that a common NMDA receptor–adenosine A_{2a} receptor–cAMP signaling cascade might be an important mechanism responsible for mediating parkinsonian symptoms.

Key words: striatum; NMDA receptors; adenosine A_{2a} receptors; cAMP; 6-OHDA; Parkinson's disease

For >30 years, dopamine replacement has formed the basis of the majority of symptomatic treatments for Parkinson's disease (Cotzias et al., 1969; Rascol et al., 1979). Because long-term use of dopamine-replacing agents is associated with severely disabling side effects, most notably dyskinesia (Quinn et al., 1987; Papa et al., 1994) or lack of maintained efficacy (Rascol, 2000), there has been increasing interest in potential non-dopaminergic treatments as monotherapies (Klockgether et al., 1994; Brotchie, 1997) or as adjuncts with L-DOPA (Wullner et al., 1992; Brotchie, 1997).

Previously, we and others have shown that overactivity of the indirect striatal output pathway connecting the striatum with the lateral segment of the globus pallidus is a key component of the neural mechanisms responsible for generating parkinsonian symptoms (Crossman et al., 1985; Pan et al., 1985; Mitchell et al., 1986; Miller and DeLong, 1987; Crossman, 1989; Mitchell and Crossman, 1989; Griffiths et al., 1990; Robertson et al., 1990; Robertson et al., 1991; Maneuf et al., 1994). The exact mechanisms driving overactivity of the indirect pathway are unclear. However, abnormal NMDA receptor transmission may be involved, because striatal NMDA receptor binding is enhanced in animal models of Parkinson's disease (Weihmuller et al., 1992; Ulas et al., 1994), and intrastriatal injection of NMDA induces parkinsonian symptoms (Klockgether and Turski, 1993). In addition, intrastriatal injection of certain NMDA receptor antagonists can reverse parkinsonism, whereas systemic administration of the NMDA receptor antagonists CP101–606, Ro 25-6981, MDL 100,453, and ifenprodil have anti-parkinsonian actions in the MPTP-lesioned primate model of

Parkinson's disease (Loschmann, 1997; Nash et al., 1997; Blanchet et al., 1999; Steece-Collier et al., 2000).

Adenosine A_{2a} receptor antagonists also have anti-parkinsonian actions when administered systemically to MPTP-lesioned primates (Kanda et al., 1998). Because within the striatum adenosine A_{2a} expression is confined to the indirect striatal output pathway, it is probable that their anti-parkinsonian actions are mediated by reducing activity of the indirect striatal output pathway (Barraco et al., 1993; Sebastiao and Ribeiro, 1996). Thus, increased activation of adenosine A_{2a} receptors may underlie, in part at least, the generation of parkinsonian symptoms. Such a possibility is suggested by the finding that intrastriatal injection of adenosine A_{2a} receptor agonists induces parkinsonism (Hauber and Munkle, 1995).

Similarities between the anti-parkinsonian actions of adenosine A_{2a} and NMDA receptor antagonists are apparent. Although both classes of compounds alleviate parkinsonism to a level comparable with those observed with dopamine replacement (Nash et al., 1997, 2000; Kanda et al., 1998), both have properties that distinguish them from “traditional” dopamine replacement therapies. For example, when administered at supraoptimal doses, neither adenosine A_{2a} receptor antagonists nor NMDA receptor antagonists elicit hyperlocomotion (Nash et al., 1997, 2000; Kanda et al., 1998), whereas dopamine-replacing agents elicit hyperactivity at supraoptimal doses in MPTP-lesioned marmosets (Pearce et al., 1995). It has also been suggested that both adenosine A_{2a} receptor antagonists and NMDA receptor antagonists can alleviate parkinsonism without eliciting dyskinesia in animals that show dyskinesia when treated with dopamine replacement therapy (Bedard et al., 1998; Blanchet et al., 1999). The studies presented here investigate a signaling mechanism common to striatal NMDA and adenosine A_{2a} receptors in normal rat striatum and in striata from the 6-hydroxydopamine (OHDA)-lesioned rat model of Parkinson's disease.

MATERIALS AND METHODS

6-OHDA lesions and sham operations. Male Sprague Dawley rats (260–280 gm, Charles River) were treated with pargyline (5 mg/kg, Sigma, St. Louis,

Received June 8, 2000; revised July 18, 2000; accepted Aug. 2, 2000.

We thank the Medical Research Council (UK) for supporting the studies reported and Paula Ravenscroft for technical assistance with HPLC.

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Figure 1. Schematic representation of region of striatum blocked to obtain slices for cAMP measurement. The area contained within the solid black lines indicates the area of striatum used for all experiments. The dotted line indicates the limits of rostral and caudal striatum as defined in experiments in which slices from the rostral and caudal striatum were separated.

MO) and desipramine (25 mg/kg, Sigma) 30 min before being anesthetized with sodium pentobarbitone (Sagatal, 60 mg/kg, i.p., Rhone Merieux). 6-OHDA-HCl (2.5 μ l, 5 mg/ml in 0.1% ascorbic acid) (Sigma) or vehicle was infused into the right medial forebrain bundle, using routine stereotaxic procedures [coordinates: +2 mm right; –2.8 mm anterior/posterior; –9 mm dorsal/ventral to skull from Bregma, according to Paxinos and Watson (1998)]. Infusions were made manually with a 26 ga Hamilton syringe over 5 min; the needle was left in place for an additional minute before being removed. After recovery, the animals were housed in groups of six under temperature-controlled conditions (19–21°C), with 12 hr alternating light/dark cycles (lights on 8 A.M. – 8 P.M.). Food and water were available *ad libitum*. Experiments were performed a minimum of 21 d after surgery.

Slice preparation. Male Sprague Dawley rats (naïve, 200–250 gm; sham-operated/6-OHDA-lesioned, 300–500 gm, Charles River) were killed by cervical dislocation. After removal of the brain, a block of tissue (width rostrocaudally, ~7.5 mm) containing the striatum (Fig. 1) was obtained by coronal razor blade cuts. This block was fixed with cyanoacrylate glue to the stage of a Vibroslice (Camden Instruments), and coronal slices of striatum were cut at 400 μ m. The cortex was removed, and striata from each hemisphere were separated. During these procedures, the tissue was maintained at 4°C in Krebs-Heinseleit solution. For each experiment, slices from six animals were distributed into 50 ml conical flasks containing 20 ml Krebs-Heinseleit solution, so that each flask contained five to eight slices from six different animals. The flasks were warmed, gassed, and maintained at 37°C in a shaking water bath for 90 min to allow the slices to recover from cutting before experimental manipulations. In experiments, which used slices prepared from 6-OHDA-lesioned or sham-operated animals to allow comparison between slices prepared from the striatum contralateral and ipsilateral to the operation, slices from the two hemispheres were distributed into separate flasks. Each experiment was repeated six times.

5'-(*N*-cyclopropyl)carboxamidoadenosine (CPCA) (0.1–100 μ M, Sigma) and 3,7-dimethyl-1-propargylxanthine (DMPX) (100 μ M, RBI, Natick, MA) were dissolved in Krebs-Heinseleit solution containing (in mM): NaCl 118, KCl 4.8, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11, and CaCl₂ 2, gassed with 5% CO₂ and 95% O₂, pH 7.4, to the desired concentration. The phosphodiesterase inhibitor, Ro 20-1724 (100 μ M, RBI) was dissolved in 100% ethanol to a concentration of 25 mg/ml and diluted in Krebs-Heinseleit solution to the required concentration. NMDA (10–300 μ M, Sigma) and 7-chlorokynureate (1.3–100 μ M, Sigma) were dissolved in NaOH and diluted as required in Krebs-Heinseleit solution to a final concentration of 0.1% NaOH.

Ro 20-1734 (100 μ M) was added along with antagonists or appropriate vehicle, by addition of small (>50 μ l) volumes of solutions to the appropriate flask. Twenty minutes after addition of Ro 20-1734, slices were

removed from the flasks and plunged into boiling sodium azide acetate buffer (10 mM) (Amersham, Arlington Heights, IL) containing 4 mM EDTA for 10 min and homogenized by sonication with a probe sonicator (Bandelin Sonoplus model HD 70). CPCA and NMDA were added 15 and 5 min, respectively, before striatal slices were removed from the flask and placed in acetate buffer.

Protein and cAMP determination. A 5 μ l aliquot of the homogenate was removed and diluted with 50 μ l of distilled water for the protein assay. Protein content was determined by the Bradford method (Bio-Rad kit) (Bradford, 1976) and Lambda Bio spectrometer (Perkin-Elmer, Norwalk, CT). Bovine serum albumin (10–200 μ g/ml) was used to generate a standard curve. The remaining homogenate from each slice was centrifuged at 10,000 $\times$ *g*_{av} for 10 min in a 1.5 ml microfuge tube (Eppendorf), and the supernatant was assayed for cAMP by scintillation proximity assay (kit reference: RPA 538, Amersham) following the manufacturer's nonacetylation method. A standard curve for cAMP concentrations ranged from 0.1 to 12.8 pmol per tube.

Lesion assessment. Subsequent to preparation of striatal slices, a single striatal slice was taken from each hemisphere of sham-operated and 6-OHDA-lesioned animals and stored at –20°C. Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in slices from each hemisphere were quantified using HPLC with electrochemical detection [method modified from Marsden and Joseph (1989)]. Each striatal slice was homogenized in 200 μ l 0.1 M perchloric acid containing 2 mM glutathione, then centrifuged at 27,000 $\times$ *g*_{av} for 20 min at 4°C (centrifuge: Howe 3K30, Sigma). A 100 μ l sample of the supernatant was filtered through 0.2 μ m Acrodiscs (Whatman, Beckman) and injected directly (autosampler: 507e, System Gold, Beckman) onto a Hypersil 5 μ m ODS-25F RP-HPLC column [flow rate of 1 ml/min (mobile phase: 12% methanol, 0.15 M NaH₂PO₄, 0.1 mM EDTA, 0.5 mM octanesulfonic acid in double-distilled deionized water); all reagents were HPLC grade (Sigma); pump: Programmable Solvent Module 126, Beckman]. Current at a potential of +0.55 V was applied to carbon-based electrodes arranged in parallel (BAS Dual Amperometric LC-4B detection system). Peaks for dopamine and DOPAC were identified by retention time using System Gold Nouveau software (Beckman). Dopamine and DOPAC levels were quantified from pre-run standard curves and expressed as microgram per milligram of wet tissue. After determination of dopamine and DOPAC levels for each experiment (each including six animals), where individual striatal slices showed <85% depletion in the side ipsilateral to the 6-OHDA-induced lesion, experiments were excluded from the study.

Data analysis. Data were expressed as percentage mean picomole cAMP per milligram of protein $\pm$ SEM of basal and were normalized to the respective basal values in each experiment. In all experiments, for each condition, the mean $\pm$ SEM shown in the Figures represents mean value of cAMP levels from six experiments for that condition (each experiment using six animals; cAMP levels measured in each of five to eight striatal slices). Statistical comparisons were made using ANOVA or Student's paired *t* test where appropriate. Where ANOVA yielded significance, *post hoc* analysis was performed using Dunnett's multiple comparisons tests or Tukey Kramer's multiple comparisons test.

RESULTS

To determine the incubation period required for NMDA to induce a maximal stimulation of cAMP levels within the striatum, striatal slices were exposed to NMDA (100 μ M) for 2–20 min. Incubation of slices with NMDA (100 μ M) at different times had a significant effect on cAMP levels compared with basal [$F_{(5,30)} = 8.5$; $p < 0.01$, ANOVA ($n = 6$)]. The maximal effect of NMDA on cAMP levels was observed after incubation for 20 min ($320.0 \pm 50.0\%$ of basal), although increases were significant compared with basal postincubation with slices for 5–20 min ($p < 0.01$ at 5, 10, 20 min; $p < 0.05$ at 18 min, *post hoc* Dunnett's multiple comparisons test) (Fig. 2A).

Incubation of striatal slices with NMDA caused a concentration-dependent increase in cAMP levels [$F_{(4,25)} = 8.9$, $p < 0.01$, ANOVA ($n = 6$)]. The maximal effect of NMDA was observed at 100 μ M ($326.2 \pm 50\%$), although increases were significant, compared with basal, at concentrations of 30–300 μ M ($p < 0.01$ at 30–100 μ M, $p < 0.05$ at 300 μ M, *post hoc* Dunnett's multiple comparisons test) (Fig. 2B).

Previous incubation of slices with 7-chlorokynureate (1.3–100 μ M; NMDA antagonist active at the glycine site) produced a concentration-dependent inhibition of NMDA-induced increases in cAMP [$F_{(6,21)} = 4.15$, $p < 0.001$, ANOVA ($n = 6$)] (Fig. 2C). There was a significant difference between striatal cAMP levels in slices incubated with 7-chlorokynureate (3.7 μ M) and NMDA compared with NMDA alone ($p < 0.05$, *post hoc* Tukey Kramer's multiple comparisons test).

Incubation of striatal slices with the A_{2a} receptor agonist CPCA caused a concentration-dependent increase in cAMP

Figure 2. Effect of NMDA on striatal cAMP. *A*, cAMP levels were measured in striatal slices subsequent to exposure to NMDA (100 μ M) at various time points between 2 and 20 min. *B*, cAMP levels were measured in striatal slices subsequent to exposure to NMDA (0–300 μ M) (5 min). *C*, cAMP levels were measured in striatal slices subsequent to exposure to NMDA (100 μ M) (5 min) that was preincubated with the glycine site NMDA receptor antagonist 7-chlorokynurenate (1.3–100 μ M). Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with ~50–60 striatal slices taken from six individual animals. * p < 0.05; ** p < 0.01; *** p < 0.001; cf. basal, *post hoc* Dunnett's multiple comparisons test.

Figure 3. Effect of adenosine A_{2a} receptor stimulation on cAMP levels. *A*, cAMP levels were measured in striatal slices subsequent to exposure to the adenosine A_{2a} receptor agonist CPCA (0.1–100 μ M) (15 min). *B*, cAMP levels were measured in striatal slices subsequent to exposure to the adenosine A_{2a} receptor agonist CPCA (3 μ M) or CPCA after preincubation with the adenosine A_{2a} receptor antagonist DMPX (100 μ M). Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with ~50–60 striatal slices taken from six individual animals. * p < 0.05, ** p < 0.01 (*A*); *post hoc* Dunnett's multiple comparisons test (*B*); *post hoc* Tukey Kramer's multiple comparisons test.

levels [$F_{(8,45)} = 3.9$, p < 0.01, ANOVA followed by *post hoc* Dunnett's multiple comparisons test ($n = 6$)]. The maximal effect of CPCA was observed at 10 μ M (579 $\pm$ 167%), although increases were significant, compared with basal, at concentrations of 3–100 μ M (p < 0.01 at 10 and 100 μ M, p < 0.05 at 3 and 30 μ M) (Fig. 3*A*).

In a separate study, the adenosine A_{2a} receptor agonist CPCA (3 μ M) significantly increased cAMP levels to 395.7 $\pm$ 67.2% of basal, which was comparable with the effect described above [$F_{(2,15)} = 10.45$; p < 0.01, ANOVA ($n = 6$)]. This increase was inhibited when slices were preincubated with the adenosine A_2 receptor antagonist DMPX (100 μ M) (p < 0.05 *post hoc* Tukey Kramer's multiple comparisons test) (Fig. 3*B*).

Previous incubation of slices with DMPX (100 μ M) blocked the NMDA receptor-mediated increase in cAMP [$F_{(2,15)} = 20.46$; p < 0.001, ANOVA ($n = 6$)]. There was a significant difference between striatal cAMP levels in slices incubated with DMPX and NMDA, compared with NMDA alone (p < 0.001, *post hoc* Tukey Kramer's multiple comparisons test) (Fig. 4).

Incubation of striatal slices with a CPCA (100 μ M) and NMDA (100 μ M) induced a dramatic increase in cAMP levels (534 $\pm$ 73% of basal, p < 0.001), which was comparable with incubation of slices with CPCA (100 μ M) alone (538 $\pm$ 43% of basal, p < 0.001) [$F_{(2,15)} = 137.6$, p < 0.001, ANOVA, *post hoc* Tukey multiple comparisons ($n = 6$)] (data not shown).

In striatal sections prepared from the unlesioned side of 6-OHDA-lesioned rats, mean dopamine and DOPAC levels were 31.6 $\pm$ 2.7 and 117 $\pm$ 17.4 ng/mg protein, respectively. Mean dopamine and DOPAC levels in striatal sections prepared from the lesioned side were 1.8 $\pm$ 0.4 and 16.7 $\pm$ 7.0 ng/mg protein, respectively. Thus, in striatal sections prepared from the lesioned striatum of 6-OHDA-lesioned animals, there was a >98% depletion of dopamine compared with the striatum on the unlesioned side. Comparisons of the mean DOPAC/dopamine ratio from lesioned

Figure 4. Effect of blockade of adenosine A_{2a} receptors on NMDA receptor-mediated increases in cAMP levels. cAMP levels were measured in striatal slices subsequent to exposure to NMDA (100 μ M) or NMDA (100 μ M) after preincubation with DMPX (100 μ M). NMDA caused a 206.5 $\pm$ 21.89% increase in cAMP levels, which was completely blocked by DMPX. Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with ~50–60 striatal slices taken from six individual animals. *** p < 0.001 *post hoc* Tukey Kramer's multiple comparisons test.

versus unlesioned striatum of individual animals showed that there was a significant increase in the DOPAC/dopamine ratio in the striatal sections prepared from the lesioned side (17.5 $\pm$ 3.1), compared with the unlesioned side 3.7 $\pm$ 0.8 (p < 0.001, Student's paired t test).

In this study, striatal slices prepared from 6-OHDA-lesioned and sham-operated rats were incubated with either NMDA or NMDA and DMPX. Three-way ANOVA using operation, side of injection, and drug treatment as factors showed significant effects of operation

tioned side compared with the lesioned side in 6-OHDA rats ($p < 0.01$, Student's t test). There was no significant difference between sides in the increase in cAMP levels induced by NMDA in sham-operated animals. Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with 50–60 striatal slices taken from six individual animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = no significance *post hoc* Tukey Kramer's multiple comparisons test.

and drug, side of injection, but no significant interaction between the three groups [$F_1(\text{operation}) = 3.52$, $p < 0.0001$; $F_1(\text{side}) = 1.49$, $p < 0.05$; $F_2(\text{drug-treatment}) = 14.87$, $p < 0.0001$, $F_1(\text{interaction} \times \text{operation} \times \text{side}) = 0.024$, $p > 0.05$; $F_2(\text{interaction operation} \times \text{drug-treatment}) = 0.94$, $p > 0.05$; $F_2(\text{interaction side} \times \text{drug-treatment}) = 0.86$, $p > 0.05$; $F_2(\text{interaction operation} \times \text{side} \times \text{drug-treatment}) = 0.357$, $p > 0.05$] ($n = 18$). Therefore, *post hoc* comparisons were made between operation and drug treatment and within a given state for drug treatment.

In striatal slices prepared from 6-OHDA-lesioned rats, basal cAMP levels in slices taken from the lesioned (dopamine-depleted) side were not significantly different from those taken from the operated side of sham-operated rats (6-OHDA-lesioned: 15.18 ± 2.54 pmol cAMP/mg protein; cf. sham-operated: 23.31 ± 4.14 pmol cAMP/mg protein, $p < 0.05$, Student's t test). In striatal slices prepared from the unlesioned side of 6-OHDA-lesioned rats, basal cAMP levels were not significantly different from those prepared from the unoperated side of sham-operated rats (unlesioned: 20.36 ± 3.52 pmol cAMP/mg protein; cf. sham-unoperated: 19.06 ± 2.05 pmol cAMP/mg protein).

There was a significant increase in striatal cAMP levels after incubation of striatal slices prepared from the operated side of sham-operated and 6-OHDA-lesioned rats with NMDA ($100 \mu\text{M}$), compared with basal (221.0 ± 28.0 and $349.6 \pm 40.0\%$, respectively) ($p < 0.01$ and $p < 0.001$, respectively, Tukey multiple comparisons test). This NMDA-induced increase in cAMP levels was greater in striatal slices prepared from the lesioned side of 6-OHDA-lesioned rats compared with the operated side of sham-operated animals ($p < 0.05$). (Fig. 5a). The NMDA-induced increase in cAMP was completely blocked after preincubation with DMPX ($100 \mu\text{M}$) in striatal slices from the operated side of both the sham-operated and 6-OHDA-lesioned animals ($p < 0.05$ and $p < 0.001$, respectively).

After incubation of slices prepared from the striatum on the unoperated side of sham-operated and 6-OHDA-lesioned animals, there was a significant effect of NMDA ($100 \mu\text{M}$) on striatal cAMP levels compared with basal (209.0 ± 23.1 and $200.9 \pm 21.9\%$, respectively) ($p < 0.05$, Tukey multiple comparisons test). However, there was no significant difference in the effect of NMDA on cAMP levels in striatal slices prepared from the unoperated side of 6-OHDA-lesioned animals, compared with the unoperated side of sham-operated animals (Fig. 5b).

CPA caused a significant increase in cAMP levels in striatal slices prepared from both the lesioned and unlesioned side of 6-OHDA-lesioned animals. Two-way ANOVA using treatment and side of injection as factors showed a significant effect of drug but not side of injection or any interaction between the two [$F(\text{drug}) = 36.4$, $p < 0.0001$, $F(\text{side of injection}) = 0.12$, $p > 0.05$, $F(\text{interaction between the two}) = 0.12$, $p > 0.05$]. Incubation of striatal slices from the

Figure 5. Effect of NMDA receptor stimulation on striatal cAMP levels in 6-OHDA rats: blockade by DMPX. Operated (a) and unoperated (b) side of 6-OHDA-lesioned and sham-operated animals. cAMP levels were measured in striatal slices subsequent to incubation with NMDA ($100 \mu\text{M}$) or NMDA and adenosine A_{2A} receptor antagonist DMPX ($100 \mu\text{M}$) and compared with basal. In slices prepared from the striatum on the operated side of sham-operated and 6-OHDA-lesioned rats, NMDA caused a 221.0 ± 28.0 and $349.6 \pm 40.0\%$ increase in cAMP levels, respectively. In striatal slices prepared from the unoperated side of sham-operated and 6-OHDA-lesioned rats, NMDA caused a 209.0 ± 23.1 and $200.9 \pm 21.9\%$ increase in cAMP levels, respectively. There was a significant difference between the increase in cAMP levels induced by NMDA in the un-

Figure 6. Effect of adenosine A_{2A} receptor stimulation on striatal cAMP levels in 6-OHDA rats. cAMP levels were measured in striatal slices prepared from the lesioned or unlesioned side of 6-OHDA rats subsequent to exposure to the adenosine A_{2A} receptor agonist CPA ($3 \mu\text{M}$) or CPA ($3 \mu\text{M}$) and the adenosine A_{2A} receptor antagonist DMPX ($100 \mu\text{M}$). In striatal slices prepared from the lesioned and unlesioned side of 6-OHDA-lesioned rats, CPA caused a 815.0 ± 205.6 and $736.8 \pm 89.3\%$ increase in cAMP levels, respectively. Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with 50–60 striatal slices taken from six individual animals. ** $p < 0.01$; cf. basal, *post hoc* Tukey Kramer's multiple comparisons test; ns, not significantly different, *post hoc* Student's t test.

dopamine-depleted side with CPA ($3 \mu\text{M}$) caused a significant increase in cAMP levels compared with basal ($815.0 \pm 205.6\%$) ($p < 0.01$, *post hoc* Dunnett's multiple comparisons test). Incubation of slices prepared from the lesioned striatum with CPA ($3 \mu\text{M}$) caused a similar rise in cAMP levels compared with basal ($736.8 \pm 89.3\%$) ($p < 0.01$, *post hoc* Dunnett's multiple comparisons test) (Fig. 6).

In striatal slices prepared from the 6-OHDA-lesioned rat, there was a significant effect of rostrocaudal level on the effect of NMDA ($100 \mu\text{M}$) on cAMP levels ($F_{(3,34)} = 4.77$, $p < 0.01$, ANOVA). In slices prepared from the rostral striatum on the lesioned side of 6-OHDA-lesioned animals, NMDA-induced increases in cAMP levels were significantly higher than basal ($726.0 \pm 329.8\%$) ($p < 0.001$). In contrast, there was no significant effect of NMDA on striatal cAMP levels prepared from the caudal striatum of the lesioned side of 6-OHDA-lesioned rats ($261.5 \pm 47.8\%$) (Fig. 7).

DISCUSSION

In this study, we show that NMDA increases striatal cAMP levels via a mechanism involving adenosine A_{2A} receptors. This signaling cascade is enhanced in the 6-OHDA-lesioned rat model of Parkinson's disease.

Figure 7. Topographical effect of NMDA on striatal slices prepared from the lesioned side of 6-OHDA-lesioned animals. cAMP levels were measured in striatal slices subsequent to exposure to NMDA (100 μ M) and compared with basal. NMDA caused a $726.0 \pm 329.8\%$ increase in cAMP compared with basal in slices prepared from the rostral striatum of 6-OHDA-lesioned animals, and a $261.5 \pm 47.8\%$ increase compared with basal in slices prepared from the caudal striatum. Data are presented as mean percentage of basal $\pm$ SEM of six separate experiments, each with 50–60 striatal slices taken from six individual animals. ** $p < 0.01$, *** $p < 0.001$, *post hoc* Tukey Kramer's multiple comparisons test.

Incubation of striatal slices with NMDA for 5–20 min significantly increased cAMP levels. Electrophysiological studies have shown that similar slice preparations are functionally viable for >20 hr (Yu et al., 1993). In this study, incubating striatal slices for 5 min with NMDA increased cAMP levels to a level comparable with those observed with longer incubation times (up to 20 min). Thus, in subsequent experiments, striatal slices were incubated with NMDA for 5 min to minimize the risk of excitotoxicity, which can occur after incubation of brain slices with NMDA (100 μ M) for periods of >30 min (Garthwaite and Garthwaite, 1986, 1989). Indeed, incubation of striatal slices with NMDA for 5 min has previously been shown to induce nontoxic, physiological changes in striatal function (Cai et al., 1991; Henselmans and Stoof, 1991).

Incubation of striatal slices with the glycine site NMDA receptor antagonist 7-chlorokynureate produced a concentration-dependent inhibition of the effect of NMDA. This illustrates that the increase in cAMP levels observed in the presence of NMDA was an NMDA receptor-mediated event. The potency of 7-chlorokynureate is comparable with that cited in other investigations using this antagonist to block NMDA receptor activation (Perrier and Benavides, 1995).

There was a concentration-dependent increase in the effect of the adenosine A_{2a} receptor agonist CPCA on cAMP levels in striatal slices. Because high concentrations of CPCA (10–100 μ M) produced variable results, subsequent experiments used CPCA (3 μ M) because this produced consistent increases in striatal cAMP levels. In addition, other studies have shown that similar concentrations of CPCA consistently activate A_{2a} adenosine receptors in brain slice preparations (Hogan et al., 1998). The CPCA-induced increase in striatal cAMP levels was inhibited when striatal slices were preincubated with the adenosine A_2 receptor antagonist DMPX, showing that the CPCA-induced increase in cAMP was mediated via adenosine A_{2a} receptors. The concentration at which DMPX inhibited the CPCA-induced increase in cAMP is comparable with that used previously to block adenosine A_{2a} receptor activation *in vitro* (Hogan et al., 1998).

NMDA receptor-mediated increases in cAMP are mediated via adenosine A_{2a} receptors

The NMDA-induced increase in striatal cAMP was completely blocked in the presence of DMPX. Although DMPX can antagonize adenosine A_{2a} and A_{2b} receptors, it is unlikely that DMPX is blocking the NMDA-induced increase in cAMP levels via blockade of adenosine A_{2b} receptors, because functional A_{2b} receptors are not expressed in rat striatum (Hide et al., 1992). Thus, the simplest explanation of the data is that NMDA receptor activation may lead to increased cAMP via indirect activation of adenosine A_{2a} receptors. If there really were a single NMDA– A_{2a} –cAMP cascade, NMDA should not increase the effect of maximal adenosine A_{2a} receptor stimulation. Thus, the finding that incubation of a supramaximal concentration of CPCA with NMDA induces a similar increase to a supramaximal concentration of CPCA alone substantiates this explanation.

cAMP is formed from ATP subsequent to activation of the membrane-bound enzyme, adenylyl cyclase. Activation of adenylyl cyclase types I–IV, VII, and VIII (Mons et al., 1995) is calcium dependent, whereas adenylyl cyclase types V, VI, and IX are activated by G_s (Mons and Cooper, 1994; Antoni et al., 1998). Because Ca^{2+} -activated adenylyl cyclase has not been detected within the striatum, the NMDA-induced increase in cAMP is unlikely to be caused by NMDA receptor-mediated elevation of intracellular calcium activating adenylyl cyclase. Within the striatum, adenylyl cyclase type V is the most abundant adenylyl cyclase, and this is activated via G_s , subsequent to adenosine A_{2a} receptor stimulation (Mons et al., 1995; Chern et al., 1996). Because the NMDA-induced increase is completely blocked in the presence of DMPX, the increase in cAMP observed after NMDA receptor activation is probably caused by indirect activation of adenosine A_{2a} receptors. We thus propose that NMDA and adenosine A_{2a}

Figure 8. Proposed mechanism for NMDA receptor-mediated increases in cAMP in the striatum. Activation of NMDA receptors results in increased intracellular Ca^{2+} levels. Ca^{2+} stimulates the release of 5'AMP, which is transported across the membrane via a bidirectional nucleotide transporter (Delaney and Geiger, 1998). 5'AMP is broken down to adenosine via membrane-bound ecto 5' nucleotidase. Adenosine then stimulates adenosine A_{2a} receptors, which are positively coupled to G_s . Once the adenosine A_{2a} receptor is stimulated, G_s activates adenylyl cyclase type V, which in turn triggers the breakdown of ATP to cAMP.

receptors share a common second messenger signaling cascade within the striatum, whereby stimulation of either NMDA receptors or adenosine A_{2a} receptors results in increased cAMP levels via activation of the same adenylyl cyclase (Fig. 8). Although not determined in the present study, such a signaling pathway may also be important in other regions of the brain, for example in the hippocampus, where both adenosine A_{2a} and NMDA receptors have been implicated in long-term potentiation (Collingridge et al., 1983; Kessey et al., 1997).

NMDA receptor–adenosine A_{2a} receptor signaling is enhanced in the 6-OHDA-lesioned animal model of Parkinson's disease

To determine the effect of NMDA on cAMP levels in the parkinsonian striatum, striatal slices were prepared from rats lesioned unilaterally with 6-OHDA. Although this is a unilateral model of Parkinson's disease, the pathological changes after 6-OHDA-induced lesions of the medial forebrain bundle correlate closely with alterations observed in idiopathic Parkinson's disease (Hornykiewicz, 1975). After infusion of 6-OHDA, although some compensatory changes may occur as a consequence of chronic dopamine depletion, these changes are thought to be similar to those occurring in patients with idiopathic Parkinson's disease.

In striatal slices prepared from 6-OHDA-lesioned rats, basal cAMP levels in slices taken from the lesioned side were comparable with those taken from the operated side of sham-operated animals. The NMDA-induced increase in cAMP observed in slices prepared from either side of sham-operated animals or the unlesioned side of 6-OHDA-lesioned animals was comparable with those observed in naïve animals (approximately twofold). NMDA caused almost a fourfold increase in cAMP levels in striatal slices prepared from the dopamine-depleted side of 6-OHDA rats. In the dopamine-depleted striatum, the enhanced effect of NMDA on cAMP levels was completely blocked by DMPX. Thus, in the dopamine-depleted striatum, the enhanced effect of NMDA on cAMP levels must be a consequence of increased signaling of the NMDA receptor–adenosine A_{2a} receptor–cAMP signaling cascade, rather than additional NMDA receptor interactions with other neurotransmitter systems. The mechanism underlying enhanced NMDA receptor stimulation in the parkinsonian striatum *in vivo* is unknown, although enhanced activation of AMPA receptors may be involved, because AMPA receptor activation has been shown to increase levels of NMDA receptor activation (Bliss and Collingridge, 1993). Furthermore, AMPA receptor antagonists elicit anti-parkinsonian actions in animal models of Parkinson's disease (Loschmann et al., 1991).

Because the effect of NMDA on cAMP levels was enhanced in the dopamine-depleted striatum, the finding that basal cAMP levels were unchanged in the lesioned striatum is perhaps surprising, because one might expect endogenous excitatory amino acids to have an effect similar to that of NMDA. One possible explanation is that transmission by endogenous excitatory amino acids within the striatum is quiescent after the recovery period of slices after preincubation. Alternatively, endogenous excitatory amino acids within the striatum may have been broken down or taken up during the recovery period. Thus, increases in basal cAMP might be seen if levels were assessed immediately postmortem or *in vivo*.

When striatal slices prepared from the 6-OHDA-lesioned striatum were incubated with the A_{2a} receptor agonist CPCA, cAMP levels were increased to the same extent as observed in the dopamine-innervated (unlesioned) striatum. Therefore, enhanced NMDA receptor–adenosine A_{2a} receptor–cAMP signaling in the dopamine-depleted striatum must be a consequence of changes occurring at the level of the NMDA receptor rather than adenosine A_{2a} receptor stimulation.

The concept that dopamine depletion results in changes in the properties of striatal NMDA receptors is in line with other data obtained in rat models of Parkinson's disease. Receptor–radioligand binding studies using washed striatal membranes in the presence of [3 H] MK-801 have shown that NMDA receptors in the

parkinsonian striatum have increased “activatability” in the presence of glutamate and glycine (Nash et al., 1997, 1999). Increased phosphorylation of NMDA receptors may account for striatal NMDA receptors becoming more activatable (Menegoz et al., 1995; Oh et al., 1998, 1999).

NMDA receptor–adenosine A_{2a} receptor signaling is enhanced specifically in the rostral striatum in the 6-OHDA-lesioned animal model of Parkinson's disease

There was a significant effect of rostrocaudal level on the ability of NMDA to increase cAMP levels in striatal slices prepared from the 6-OHDA-lesioned striatum. NMDA only induced significant increases in striatal cAMP levels in striatal slices prepared from the rostral striatum on the lesioned side of 6-OHDA-lesioned animals. This topographical specificity is in line with previous findings in which blockade of NMDA receptors specifically within the rostral striatum mediated anti-parkinsonian actions, whereas blockade of NMDA receptors in more caudal regions had no effect on locomotion (Klockgether and Turski, 1993; Nash et al., 1997). Indeed, anatomical tracing and pharmacological and immunohistochemical studies have shown that both receptor density and neurotransmitter content, as well as afferent and efferent connections, vary between the rostral and caudal striatum (Beckstead and Cruz, 1986; Widmann and Sperk, 1986; Albin et al., 1992; Kincaid and Wilson, 1996).

Enhanced NMDA receptor–adenosine A_{2a} receptor–cAMP signaling on the indirect striatal output pathway is responsible for the induction of parkinsonian symptoms

Overactivity of the indirect striatal output pathway is thought to be the key mechanism responsible for the induction of symptoms of Parkinson's disease (Crossman et al., 1985; Pan et al., 1985; Mitchell et al., 1986; Miller and DeLong, 1987; Crossman, 1989; Mitchell and Crossman, 1989; Griffiths et al., 1990; Robertson et al., 1990, 1991; Maneuf et al., 1994). Although NMDA receptors are expressed on both the direct and indirect pathway, as well as on glia (Thompson et al., 2000), the selective location of adenosine A_{2a} receptors on the indirect pathway (Barraco et al., 1993; Sebastiao and Ribeiro, 1996) makes it probable that NMDA receptor–adenosine A_{2a} receptor–cAMP signaling occurs specifically on the indirect pathway. Furthermore, NMDA receptor antagonists are thought to mediate their anti-parkinsonian actions specifically via the indirect pathway, suggesting that NMDA receptors on this pathway are overactive in Parkinson's disease (Schmidt et al., 1990, 1992).

In conclusion, we have demonstrated that within the striatum, NMDA receptors modulate cAMP levels via adenosine A_{2a} receptors within the striatum. Furthermore, this NMDA receptor–adenosine A_{2a} receptor–cAMP signaling cascade is enhanced in the dopamine-depleted striatum. Both this enhancement and the anti-parkinsonian action of NMDA receptor antagonists are restricted to the rostral striatum, suggesting that blockade of this overactive signaling cascade may account for the anti-parkinsonian actions of NMDA receptor antagonists and adenosine A_{2a} receptor antagonists in MPTP-lesioned primates. At present, it is not clear how NMDA–adenosine A_{2a} receptor signaling is enhanced, although adenosine A_{2a} receptor function appears normal.

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