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Persistent Behavioral Sensitization to Chronic L-DOPA Requires A_{2A} Adenosine Receptors

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To investigate the role of A_{2A} adenosine receptors in adaptive responses to chronic intermittent dopamine receptor stimulation, we compared the behavioral sensitization elicited by repeated L-DOPA treatment in hemiparkinsonian wild-type (WT) and A_{2A} adenosine receptor knock-out (A_{2A} KO) mice. Although the unilateral nigrostriatal lesion produced by intrastriatal injection of 6-hydroxydopamine was indistinguishable between WT and A_{2A} KO mice, they developed strikingly different patterns of behavioral sensitization after daily treatment with low doses of L-DOPA for 3 weeks. WT mice initially displayed modest contralateral rotational responses and then developed progressively greater responses that reached a maximum within 1 week and persisted for the duration of the treatment. In contrast, any rotational behavioral sensitization in A_{2A} KO mice was transient and completely reversed within 2 weeks. Similarly, the time to reach the peak rotation was progressively shortened in WT mice but remained unchanged in A_{2A} KO mice. Furthermore, daily L-DOPA treatment produced gradually sensitized grooming in WT mice but failed to induce any sensitized grooming in A_{2A} KO mice. Finally, repeated L-DOPA treatment reversed the 6-OHDA-induced reduction of striatal dynorphin mRNA in WT but not A_{2A} KO mice, raising the possibility that the A_{2A} receptor may contribute to L-DOPA-induced behavioral sensitization by facilitating adaptations within the dynorphin-expressing striatonigral pathway. Together these results demonstrate that the A_{2A} receptor plays a critical role in the development and particularly the persistence of behavioral sensitization to repeated L-DOPA treatment. Furthermore, they raise the possibility that the maladaptive dyskinetic responses to chronic L-DOPA treatment in Parkinson's disease may be attenuated by A_{2A} receptor inactivation.

Key words: A_{2A} adenosine receptor; L-DOPA; behavioral sensitization; Parkinson's disease; dyskinesia; dynorphin

For >30 years the dopamine precursor L-DOPA has been the most effective and commonly prescribed treatment for Parkinson's disease (PD). Despite its considerable symptomatic motor benefit, chronic administration of L-DOPA leads to abnormal motor responses known as dyskinesias, involving involuntary choreic or dystonic movements in >50% of patients (5 years after the initiation of the treatment) (Chase, 1998; Obeso et al., 2000). Such shortcomings of L-DOPA and other dopaminergic drugs have prompted a search for alternative treatment strategies that provide symptomatic benefits while avoiding the delayed motor complications associated with the long-term use of antiparkinsonian drugs. Several neurotransmitters have been implicated in the motor complications elicited by repeated dopamine receptor stimulation, including glutamate (Marin et al., 1996; Tzschentke and Schmidt, 1998; Calabresi et al., 2000), cannabinoids (Souilhac et al., 1995; Zeng et al., 1999), opioids (Henry and Brotchie, 1996), and adenosine (Richardson et al., 1997; Kanda et al., 2000; Jenner, 2000). Recently, the A_{2A} adenosine receptor has emerged as an attractive target for PD treatment by virtue of its concentrated expression in striatopallidal neurons and its modulation of dopamine receptor-mediated functions (Schiffmann et al., 1991; Fink et al., 1992; Ferré et al., 1997; Svenningsson et al., 1999). In addition to the documented motor-activating feature of A_{2A} receptor antagonists (Richardson et al., 1997; Impagnatiello et al., 2000), their minimal propensity for eliciting dyskinesia in L-DOPA-primed nonhuman primates (Kanda et al., 1998; Grondin et al., 1999) has further enhanced their therapeutic potential in PD.

To critically evaluate the involvement of A_{2A} receptors in L-DOPA-induced dyskinesia, we have adopted a rodent "priming" model in which behavioral sensitization is elicited by repeated treatment with a low dose of L-DOPA in unilateral 6-hydroxydopamine (6-OHDA)-lesioned mice (Carey, 1991; Henry et al., 1998). The delayed induction of markedly increased contralateral rotation as well as several characteristic neurochemical adaptations in this rodent model closely resemble several behavioral and neurochemical features of L-DOPA-induced dyskinesia in parkinsonian nonhuman primates and in PD patients (Brotchie, 1998; Henry et al., 1998). Thus, this behavioral sensitization model may provide useful information on the maladaptive neuronal plasticity that underlies L-DOPA-induced dyskinesia in PD. We examined the effect of genetic inactivation of A_{2A} receptors on chronic L-DOPA-induced behavioral sensitization using this rodent model of dyskinesia. Our genetic (knock-out) approach to A_{2A} receptor inactivation offers several advantages

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that complement standard pharmacological approaches, which are hindered by intrinsic limitations of partial specificity and efficacy. The results suggest that the A_{2A} receptor is required for the development and persistence of L-DOPA-induced behavioral sensitization in mice.

MATERIALS AND METHODS

Breeding of A_{2A} adenosine receptor knock-out mice

The A_{2A} adenosine receptor knock-out (A_{2A} KO) mouse line was generated in a pure 129-Steel genetic background as previously described (Chen et al., 1999a) and was maintained for >3 years through breeding by heterozygote intercrosses. For each of the present experiments, wild-type (WT) and A_{2A} KO littermates (both male and female) of the F4 generation were matched for gender, age (4–5 months), and body weight (22–27 gm).

6-Hydroxydopamine lesions

All experiments were performed in accordance with Massachusetts General Hospital and National Institutes of Health Guidelines on the ethical use of animals. They were maintained in "home" cages with a 24 hr 1:1 light/dark cycle. To produce unilateral striatal dopamine depletion, the mice were pretreated with desipramine hydrochloride (25 mg/kg), to minimize damage to noradrenergic neurons. Under Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol) anesthesia (20 ml/kg, i.p.), mice were infused with 10 μg of 6-OHDA (2.5 μg/μl in normal saline containing 0.05% of ascorbic acid) delivered by a microinfusion pump (1 μl/min) into the left dorsal striatum at the following coordinates (from bregma: 1.1 mm anterior, 1.5 mm lateral, 2.0 mm ventral) (Franklin and Paxinos, 1997).

Behavioral analysis

Behavioral analysis was performed by an observer, who was blinded to the genotype of the animals. Seven days after 6-OHDA lesioning, the WT and A_{2A} KO mice (n = 9–12) were randomly assigned to daily treatment with either low doses of L-DOPA (1.0, 1.8, and 2.5 mg/kg) or water for 3 weeks. All animals were pretreated with benserazide, a peripheral decarboxylase inhibitor (2.0 mg/kg, i.p., 20 min before L-DOPA or water injection). Contralateral rotation and grooming behaviors were evaluated in a test cage every other day from day 1 to day 14, and every 2 d from day 15 to day 20. The intensity and kinetic profile of L-DOPA-induced contralateral behavior was monitored, and recordings were established by monitoring the number of complete (360°) rotations ipsilateral and contralateral to the lesion in a 60 min testing period immediately after the injection of L-DOPA or water. Stereotyped grooming behavior was scored (in a 60 min test period immediately after the injection) by the observer using the following scale: 0 = inactive (mostly motionless without stereotypies), 1 = active without grooming (coordinated movements/exploration without stereotypies), 2 = mild grooming (sporadic face washing and head stretching), 3 = moderate grooming (frequent but discontinuous face washing, rearing and standing with hind limbs), 4 = vigorous grooming (repetitive face and body washing in a sequential chain of grooming activity), and 5 = intensive grooming (grooming of any kind with forepaws interspersed with vigorous grooming of the hindflank or anogenital region).

Biochemical assessments

Striatal content of DA and DOPAC. In a separate experiment, 7 d after 6-OHDA injection, mice were killed by rapid cervical dislocation, and their striata were dissected and assayed for catecholamines by standard reverse-phase HPLC with electrochemical detection, as described previously (Chen et al., 2001).

Dopamine transporter binding autoradiography. Dopamine transporter (DAT) binding was assessed using the radioligand [^{3}H]-mazindol (specific activity = 24 Ci/mmol; DuPont NEN, Boston, MA) in mice killed 1 week after 6-OHDA injection (and before any L-DOPA treatment) or 4 weeks after the 6-OHDA injection (i.e., 3 weeks after the first daily L-DOPA treatment and 24 hr after the last L-DOPA treatment). Twenty micrometer coronal sections at the levels of anterior, middle, and posterior striatum were processed for [^{3}H]-mazindol binding as previously described (Chen et al., 2001). Specific striatal [^{3}H]-mazindol binding (femtomoles per milligram of tissue) was calculated by subtracting nonspecific binding (in the presence of 100 μM unlabeled nomifensine) from total binding.

In situ *hybridization histochemistry. In situ* hybridization histochemistry with cRNA probes was performed according to protocols described in previous studies (Moratalla et al., 1996a,b). Mouse brain sections were post-fixed in buffered 4% paraformaldehyde, acetylated in acetic anhydride, and dehydrated in graded ethanol. Sections were hybridized with a ^{35}S-labeled RNA probe (150,000 cpm/μl buffer) specific for the rat prodynorphin cDNA (provided by J. Douglass) (Civelli et al., 1985) in a hybridization buffer described previously (Moratalla et al., 1996a,b). After hybridization, sections were washed and then treated with RNase A (100 μg/ml), and washed again to final strength in 0.1× SSC at 70°C for 30 min. The slides were rinsed, dried, and exposed to BioMax MR films (Amersham Biosciences, Arlington Heights, IL) for 15–20 d.

Image analysis. Optical densities on the film were determined using a computing densitometer equipped with an image analysis program (model 300A; Molecular Dynamics, Sunnyvale, CA). Approximately three or four sections through the striatum, at rostral and middle levels, were analyzed for each mouse. For each section, dynorphin mRNA levels were determined by optical densities that were in the 6-OHDA lesioned side (left) and the contralateral side (right). Dynorphin mRNA levels from 6-OHDA lesioned side were expressed as a percentage of the contralateral (unlesioned) side because previous experiments have demonstrated that a unilateral 6-OHDA lesion did not alter dynorphin mRNA expression in the contralateral striatum (Cenci et al., 1993).

Statistical analysis

All data are expressed as group mean ± SEM and analyzed using "SAS" or "SPSS" statistical programs. The significance of differences between two genotypes across multiple treatment groups was evaluated by split-plot ANOVA for repeated measures followed by Fisher's least significant difference (LSD) comparison test. The significance of differences between responses on treatment day 1 and subsequent treatment days was evaluated by one-way ANOVA for repeated measures, followed by Dunnett's test. Selective comparison of specific treatment day versus day 1 was evaluated by the randomization test for matched pairs (Siegel and Castellan, 2000). Grooming behavioral data were analyzed by the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U test. The differences in dynorphin mRNA levels between WT and KO groups were analyzed by Student's t test.

RESULTS

Intrastriatal 6-OHDA injection produced indistinguishable dopaminergic neurotoxicity in WT and A_{2A} KO mice

Previous studies in our laboratory and others showed that inactivation of A_{2A} receptors by either genetic ablation or pharmacological blockade protects against brain injuries induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Chen et al., 2001), kainic acid (Jones et al., 1998a,b), or middle cerebral artery occlusion (Monopoli et al., 1998; Chen et al., 1999a). Accordingly, we first determined whether the dopaminergic lesion induced by 6-OHDA differs between A_{2A} KO and WT mice. The administration of 6-OHDA into the left striatum of mice significantly reduced striatal levels of dopamine as well as its main metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) ipsilateral to the lesion (Table 1). These reductions were indistinguishable between WT and A_{2A} KO mice.

The extent of the lesion was also assessed by determining the DAT binding density in striatum before and after chronic L-DOPA treatment. Seven days after the unilateral 6-OHDA injection, striatal DAT binding density decreased significantly in the ipsilateral striatum of L-DOPA-naive mice (Table 1). Consistent with the dopamine content data, the reduction of DAT binding on the ipsilateral side was virtually identical between WT and A_{2A} KO groups (Table 1). Similarly, in the mice treated with L-DOPA daily for 3 weeks, no difference in the reduction of ipsilateral DAT density was found between the two genotypes. There was also no difference between KO and WT mice with respect to the slight rise in contralateral DAT binding density observed after the repeated L-DOPA treatments. [The basis for

Table 1. Neurochemical and autoradiographic measures of nigrostriatal innervation in WT and A_{2A} KO mice after a unilateral 6-OHDA lesion

	DA	DOPAC	DAT density (optical densities × 100)	
Genotype	(pm/mg tissue)		Naive	Sensitized
Wild-type				
Ipsilateral	15 ± 3*	1.2 ± 0.2*	154 ± 15*	163 ± 21*
Contralateral	61 ± 1	4.0 ± 0.2	285 ± 27	407 ± 16
A_{2A} KO				
Ipsilateral	12 ± 3*	1.0 ± 0.2*	161 ± 19*	165 ± 20*
Contralateral	60 ± 2	3.5 ± 0.3	289 ± 35	415 ± 18

DA and DOPAC were determined in striatal homogenates obtained from WT and A_{2A} KO animals naive to L-DOPA treatment and killed 1 week after the injection of 6-OHDA. Striatal DAT densities were determined using tissue samples obtained before (naive) and after 3 weeks (sensitized) of intraperitoneal 2.5 mg/kg L-DOPA treatment.
*$p < 0.05$ versus respective contralateral side.

this rise is unclear, although a similar elevation in DAT binding density in unlesioned striatum after repeated L-DOPA treatment has been reported by others (Ikawa et al., 1993).] Thus, these results confirm that the intrastriatal injection of 6-OHDA produced similar dopaminergic neurotoxicity, setting the stage for a meaningful comparison of behavioral sensitization in hemiparkinsonian WT and A_{2A} KO mice. These data also indicate that the neuroprotective effect of A_{2A} receptor inactivation against MPTP toxicity (Chen et al., 2001) does not necessarily apply to all models of PD.

Lack of persistent rotational sensitization to chronic L-DOPA treatment in hemiparkinsonian A_{2A} KO mice

To evaluate A_{2A} receptor involvement in L-DOPA-induced behavioral sensitization, we treated WT and A_{2A} KO mice with 20 daily doses of L-DOPA beginning 1 week after unilateral intrastriatal injection of 6-OHDA. L-DOPA at the very low dose of 1.0 mg/kg, intraperitoneally, did not result in any significant contralateral or ipsilateral turning behavior in either WT and A_{2A} KO mice (Fig. 1*A*) as compared with the vehicle-treated group in which only a weak net ipsilateral response (8 ± 1 and 5 ± 5 net ipsilateral turns/hr on day 1 in WT and A_{2A} KO mice, respectively) could be seen consistently throughout the test period (data not shown). However, the administration of L-DOPA at higher but still modest doses 1.8 and 2.5 mg/kg produced a marked behavioral sensitization in WT mice (Fig. 1*B,C*, respectively). Specifically, after a lag time of 3 d, mice treated daily with 1.8 mg/kg L-DOPA began to develop an increased contralateral rotational response that reached a maximum by day 9 (Fig. 1*B*). In WT mice the sensitized response persisted unabated for the duration of the 20 d treatment period. However, the behavioral pattern induced by repeated L-DOPA treatment in A_{2A} KO mice markedly differed from that in WT mice (Fig. 1*B*) ($F_{(8,160)} = 2.92$; $p < 0.01$; split-plot ANOVA). Although A_{2A} KO mice displayed a trend of gradual enhancement of contralateral rotations reaching an apparent maximum between days 5 and 9 of daily 1.8 mg/kg L-DOPA treatment, they failed to develop statistically significant behavioral sensitization at this dosage (one-way ANOVA for repeated measurements; $n = 12$; $F_{(8,99)} = 1.677$; $p = 0.118$). Most strikingly, any enhancement of rotational response to L-DOPA in A_{2A} KO mice was not maintained after day 9. Instead, by day 14 the response to L-DOPA had returned to baseline (i.e., that on day 1) where it remained for the rest of the experiment. As previously noted and discussed, acute dopaminergic stimulation of motor activity (as on day 1) is not enhanced in A_{2A} KO mice, in contrast to the demonstrated potentiating effect of A_{2A} antagonists on motor activity (Chen et al., 2000).

Because A_{2A} KO mice primarily failed to develop significantly sensitized rotational behavior, the possibility arose that using this low dose of L-DOPA (1.8 mg/kg) a "threshold" response needed to elicit sustained behavioral sensitization was reached in WT but not KO mice. Indeed, the acute motor response to dopaminergic stimulation (e.g., on day 1) (Fig. 1*B*) may have been slightly lower in A_{2A} KO than in WT mice (as we and others have previously observed; Ledent et al., 1997; Chen et al., 2000). To address this possibility we attempted to overcome such a threshold effect by examining the influence of A_{2A} receptor inactivation on the duration of sensitization induced by a higher dose of L-DOPA. As shown in the Figure 1*C*, 2.5 mg/kg L-DOPA produced substantially greater contralateral turning (compared with 1.8 mg/kg daily) for both the initial and maximal response, with the latter occurring earlier (by day 7) using the higher dose, in both WT and A_{2A} KO mice. Nevertheless, the rotational sensitization was again maintained for the duration of daily L-DOPA treatment only in WT mice. By contrast, the response to this higher dose of L-DOPA in A_{2A} KO mice, after apparently sensitizing with a peak on day 5 ($p < 0.05$ only for day 5 compared with day 1 by the randomization test for matched pairs, and $p > 0.05$ by one-way ANOVA for repeated measurements), reverted by day 17 to that observed on day 1. Note that the absence of persistent sensitization in KO mice was as prominent at 2.5 mg/kg as it was at 1.8 mg/kg L-DOPA, although their peak rotational responses at the higher dose exceeded the peak responses observed in WT mice at the lower dose. Thus, these data confirm the effect of A_{2A} receptor inactivation on the development and particularly the maintenance of L-DOPA-induced rotational sensitization and rule out the possibility that it can be explained by nonspecific subthreshold responses to L-DOPA in A_{2A} KO mice.

Because development of L-DOPA-induced behavioral sensitization is associated with a progressively shortened onset and duration of action, we also compared how the kinetics of L-DOPA-induced responses changed over the 3 week course of daily treatments in WT and A_{2A} KO mice. As shown in Figure 2*A*, the time to reach the peak contralateral turning response to a single dose of L-DOPA (1.8 mg/kg) was progressively shortened in WT mice (from 20–30 min on day 1, to 20 min on day 11, to 10 min on day 20). In contrast, the time to reach the peak response in A_{2A} KO animals remained virtually unchanged throughout the treatment (~20 min for days 1, 11 and 20). Moreover, the number

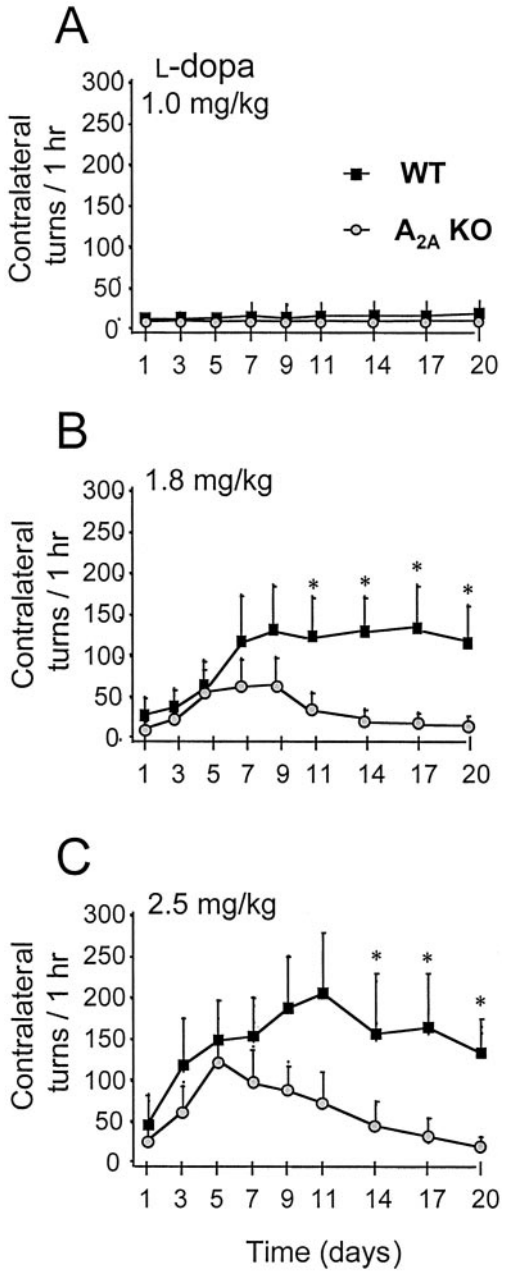

Figure 1. Effect of A_{2A} receptor deficiency on dose-dependent L-DOPA-induced behavioral sensitization in hemiparkinsonian mice. WT (*black squares*) and A_{2A} KO (*gray circles*) mice were treated with benserazide (2 mg/kg, i.p.) plus L-DOPA at intraperitoneal doses of 1.0 mg/kg (*A; n* = 10 per each genotype), 1.8 mg/kg (*B; n* = 10 WT mice and *n* = 12 KO mice), or 2.5 mg/kg (*C; n* = 10 per each genotype) once a day for 20 d. Contralateral rotational behavior was evaluated for the 1 hr test period immediately after the administration of L-DOPA on the indicated days. Data are expressed as mean ± SEM of the net contralateral rotations (contralateral minus ipsilateral turns). For *B*, $F_{(8,160)} = 2.92$, $^*p < 0.01$ compared with the corresponding KO value (split-plot ANOVA followed by Fisher's LSD comparison test); for *C*, $F_{(8,135)}=2.32$, $^*p < 0.05$ compared with the corresponding KO value (split-plot ANOVA followed by Fisher's LSD comparison test).

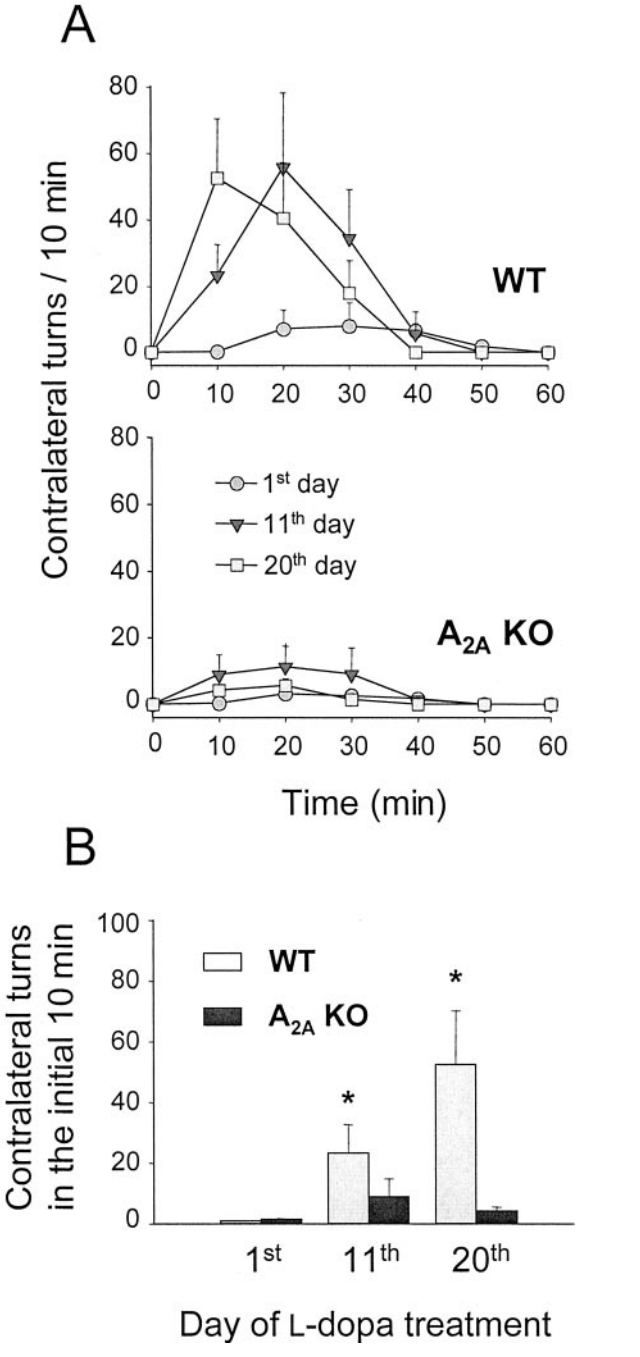

Figure 2. Peak rotational response to L-DOPA occurs progressively earlier in hemiparkinsonian WT but not A_{2A} KO mice. *A* shows the initial, midway, and final time courses for turning induced by the daily intraperitoneal administration of 1.8 mg/kg L-DOPA in WT and A_{2A} KO mice, i.e., on the 1st (*shaded circles*), 11th (*filled triangles*), and 20th (*shaded squares*) day of 20 consecutive daily L-DOPA treatments. *B* (replotted from the 10 min time points in *A*) depicts the progressive increase in contralateral rotations for the initial 10 min after L-DOPA administration from the 1st to the 11th to the 20th day in WT mice ($F_{(2,40)}=7.32$; $^*p < 0.01$; split-plot ANOVA followed by Fisher's LSD test) but not in A_{2A} KO mice.

of contralateral rotations occurring over the initial 10 min after L-DOPA administration increased steadily and significantly in WT but not A_{2A} KO mice from day 1 to day 20 (Fig. 2*B*). These results further support an important role for the A_{2A} receptor in the adaptive behavioral responses to chronic L-DOPA treatment.

Lack of sensitized grooming responses to chronic L-DOPA in hemiparkinsonian A_{2A} KO mice

We also examined the effect of A_{2A} receptor inactivation on chronic L-DOPA-induced grooming behaviors. Grooming is by its nature a stereotyped behavior, and it has been proposed to share

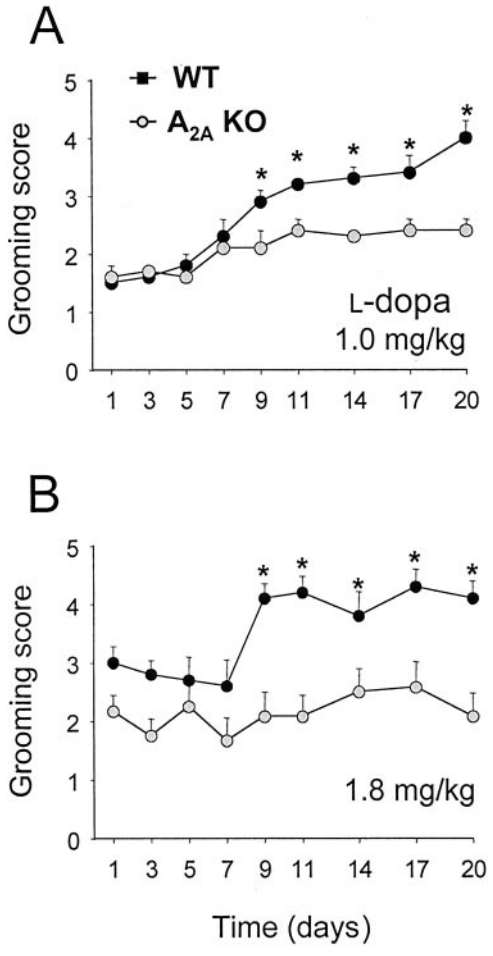

Figure 3. Sensitization of DOPA-induced grooming behavior is attenuated in mice lacking adenosine A_{2A} receptors. Grooming activity, defined as maximal grooming score recorded over the 1 hr testing period, was determined in WT (*black squares*) and A_{2A} KO (*gray circles*) animals receiving an intraperitoneal dose of 1.0 mg/kg (*A*) or 1.8 mg/kg (*B*) L-DOPA. Data are expressed as mean ± SEM of 10–12 animals. $^*p < 0.05$; compared with the corresponding KO value; Kruskal–Wallis test followed by Mann–Whitney *U* test.

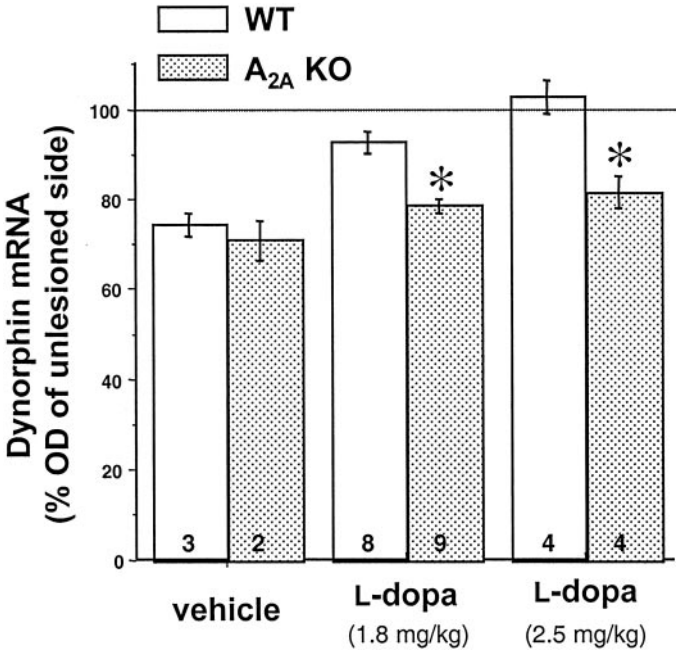

Figure 4. Daily L-DOPA reverses the reduction in dynorphin expression induced by 6-OHDA in WT but not A_{2A} KO mice. The dynorphin mRNA levels were determined by *in situ* hybridization histochemistry in mice unilaterally lesioned with 6-OHDA, followed by daily treatment with L-DOPA (1.8 or 2.5 mg/kg) or vehicle for 21 d. Dynorphin mRNA levels [optical density (OD)] were quantified at the level of midstriatum and expressed as a percentage of the contralateral side (unlesioned striatum). Chronic treatment with L-DOPA reverses the 6-OHDA-induced reduction in dynorphin mRNA to normal levels in WT but not A_{2A} KO mice ($^*p < 0.05$; Student's *t* test compared with the corresponding WT group). The *numbers* inside the *bars* indicate the animal numbers for each group.

common neurochemical features with L-DOPA-induced dyskinesia in primates (Graybiel et al., 2000). Daily treatment with vehicle did not induce any significant grooming behavior in WT and KO mice of 129-Steel genetic background (most average scores were <1, $n = 2$ for WT and $n = 3$ for KO mice, respectively; data not shown). However, daily L-DOPA administration to WT mice (Fig. 3) increased the frequency and intensity of grooming after repeated challenges, reaching its maximum after 11 d at the lowest dose tested (1.0 mg/kg) (Fig. 3*A*) or 9 d using the intermediate dose (1.8 mg/kg) (Fig. 3*B*) ($n = 10–12$; $p < 0.05$; Kruskal–Wallis test followed by Mann–Whitney *U* test). Grooming responses then remained enhanced throughout the duration of treatment (Fig. 3). In A_{2A} KO mice, by contrast, the grooming responses to either of these daily doses of L-DOPA showed no significant sensitization over the entire 3 week period (Fig. 3) ($n = 10–12$). Thus, chronic L-DOPA-induced sensitization of grooming behavior in WT but not in KO mice provides evidence for A_{2A} receptor involvement in L-DOPA-induced sensitization of stereotyped as well as rotational behaviors.

Daily L-DOPA reverses the reduction in dynorphin expression induced by 6-OHDA in WT but not A_{2A} KO mice

Finally, we also examined the effect of A_{2A} receptor inactivation on chronic L-DOPA-induced striatal neuronal activity using dynorphin mRNA as a cellular readout. Dynorphin mRNA is predominantly coexpressed with D_1 dopamine receptor mRNA in striatonigral neurons (the "direct" pathway) (Gerfen et al., 1990; Le Moine et al., 1990). The L-DOPA-induced reversal of the 6-OHDA-induced reduction in dynorphin mRNA has been associated with the development of L-DOPA-induced dyskinesia in parkinsonian animals (Andersson et al., 1999; Jenner, 2000). *In situ* hybridization analysis shows that 6-OHDA lesioning reduced dynorphin mRNA levels in the ipsilateral striatum of both WT and KO mice. Consistent with previous reports (Andersson et al., 1999; Henry et al., 1999; Pirker et al., 2001), chronic treatment with L-DOPA dose-dependently reverses the 6-OHDA-induced reduction in dynorphin mRNA to normal levels in WT mice (Fig. 4) ($n = 8–9$; $p > 0.05$; compared with contralateral striatum, paired Student's *t* test). L-DOPA did not, however, increase dynorphin mRNA above normal levels in WT mice (as others have observed; Andersson et al., 1999; Henry et al., 1999), probably because of the particularly low doses of L-DOPA tested here (1.0–2.5 mg/kg, threefold less than in the otherwise similar studies). In contrast, chronic L-DOPA treatment failed to reverse the reduction in dynorphin mRNA to normal levels in A_{2A} KO mice (Fig. 4). Thus, the A_{2A} receptor may contribute to the altered neuronal activity of the "direct" striatonigral pathway that is associated chronic L-DOPA-induced behavioral changes.

DISCUSSION

The A_{2A} adenosine receptor is required for L-DOPA-induced behavioral sensitization

The main finding of our study is that genetic inactivation of A_{2A} receptors abolished sustained behavioral sensitization in response to repeated L-DOPA treatment in mice. Our results clearly show that WT mice developed progressively enhanced contralateral rotational, whereas A_{2A} KO mice developed only a trend toward L-DOPA-induced sensitization of rotational responses that appeared milder than in WT mice and primarily failed to reach statistical significance. The time to reach peak rotation progressively shortened in WT but not A_{2A} KO mice. In keeping with the lack of sustained rotational behavioral sensitization, A_{2A} receptor inactivation also prevented or markedly reduced the induction of grooming responses. These altered behavioral responses were further substantiated by the neurochemical finding that repeated L-DOPA treatment reversed the 6-OHDA-induced reduction of striatal dynorphin mRNA levels in WT but not A_{2A} KO mice. Thus, the absence of chronic L-DOPA-induced sensitization at the behavioral (contralateral rotation and grooming) and cellular (dynorphin mRNA) levels in hemiparkinsonian mice lacking A_{2A} receptors strongly suggests that A_{2A} receptors are required for L-DOPA-induced behavioral sensitization.

The most striking finding of the present study is that 6-OHDA-lesioned mice lacking the A_{2A} receptors do not maintain sensitized rotational motor responses to repeated L-DOPA administration. Although the maintenance of behavioral sensitization, like its induction and expression, is recognized as a mechanistically discrete phase (Wolf, 1998; Chase and Oh, 2000; Li et al., 2000; Vanderschuren and Kalivas, 2000), relatively little is known about the unique molecular pathways leading to the persistence of this phenomenon, which can last for months or years under some circumstances (Robinson and Becker, 1986). The present data indicate that A_{2A} receptors may play a critical role in the maintenance of L-DOPA-induced motor sensitization. Thus, they suggest that endogenous adenosine acting on (presumably striatal) A_{2A} receptors may facilitate the long-term adaptive changes induced by chronic L-DOPA exposure.

The reversal of an apparent sensitization to L-DOPA (Fig. 1*B,C*) in the absence of functional A_{2A} receptors could be explained by the rapid development of tolerance. This consideration is prompted by the well documented phenomenon of tolerance to the motor effects of the nonspecific adenosine receptor antagonist caffeine (for review, see Fredholm et al., 1999). However, several studies have now shown that the motor activation induced by a specific A_{2A} receptor antagonist shows no tolerance after chronic intermittent administration (Halldner et al., 2000; Pinna et al., 2001). Moreover, in a preliminary study the locomotor response to self-administered intravenous cocaine showed no tolerance in A_{2A} KO mice, in contrast to the marked tolerance that develops in their WT littermates (Rocha et al., 2001). Therefore, tolerance is unlikely to account for a reversal of newly developed sensitization in A_{2A} KO mice. The absence of active A_{2A} receptor-mediated maintenance of the adaptive changes underlying sensitization may offer a better explanation.

The absence of sustained sensitization to repeated L-DOPA treatment in mice lacking the A_{2A} receptor likely reflects a broader phenotype of attenuated adaptive motor responses to intermittent dopaminergic stimulation. Indeed, we have found that the robust locomotor sensitization to the indirect dopamine agonist amphetamine in WT mice is completely absent in their A_{2A} KO littermates (Chen et al., 1999b). In line with these findings, a withdrawal syndrome that develops after chronic repeated alcohol administration was attenuated in mice lacking the A_{2A} receptor and in mice treated with the specific A_{2A} antagonist ZM 241385 (El Yacoubi et al., 2001). However, the extent to which acute pharmacological blockade of the A_{2A} receptor parallels the A_{2A} KO phenotype in preventing sensitized responses to chronic dopaminergic stimulation remains to be clarified. For example, although one study has raised the possibility that L-DOPA-induced motor sensitization may be prevented by paired exposure to a specific A_{2A} antagonist (Pinna et al., 2001), another has suggested that a specific A_{2A} agonist can have the same effect on methamphetamine-induced motor sensitization (Shimazoe et al., 2000). Nevertheless when taken together, A_{2A} receptor studies based on either pharmacological or genetic approaches have consistently pointed to an important role for this receptor in adaptive motor behaviors.

Potential mechanisms by which the A_{2A} receptor facilitates L-DOPA-induced behavioral sensitization

Considerable research effort has been devoted to clarifying the anatomical and neurochemical basis of sensitized behavioral responses to dopaminergic stimuli. Although this phenomenon has been well localized to the mesolimbic and nigrostriatal dopaminergic systems of the basal ganglia, less is clear about the specific dopaminergic receptor subtypes, as well as the other neurotransmitters and the intracellular signaling cascades mediating these adaptive changes (Pierce and Kalivas, 1997). Both D_1- and D_2-like receptors have been implicated in L-DOPA-induced sensitization (Morelli and Di Chiara, 1987; Blanchet et al., 1995, Bordet et al., 2000). Activation of A_{2A} receptors has been shown to antagonize D_2 receptor-mediated neurotransmitter release, immediate-early gene expression, and psychomotor stimulation (Ferré et al., 1997; Svenningsson et al., 1999). Thus, the A_{2A} receptor may modulate L-DOPA-induced behavioral sensitization through its cellular-level interaction with the D_2 receptor in striatopallidal neurons (of the "indirect" pathway), which express both A_{2A} and D_2 receptors. Within striatal neurons multiple intracellular signaling cascades, the cAMP pathway in particular, have also been implicated in the basal ganglia plasticity that underlies sensitization to dopaminergic stimulation (Chase and Oh, 2000; Jenner, 2000). For example, repeated treatment with L-DOPA or other dopaminergic stimuli has been shown to enhance phosphorylation and activation of cAMP pathway targets, including dopamine- and cAMP-dependent phosphoprotein (DARPP-32) and cAMP-response element binding protein and expression of the long half-life transcription factor FosB (Barone et al., 1994; Moratalla et al., 1996a; Pierce and Kalivas, 1997; Chase and Oh, 2000; Dunah et al., 2000). The A_{2A} receptor, which is positively coupled to adenylate cyclase and cAMP production through G_s (Svenningsson et al., 1999), may affect L-DOPA-induced neurochemical and behavioral changes by influencing this pathway. In this regard, a recent study shows that D_2 receptor blockade induces phosphorylation of DARPP-32 protein in WT but not A_{2A} KO mice, indicating a critical role for A_{2A} receptors in striatal cellular signaling involving the cAMP pathway (Svenningsson et al., 2000). Further studies are needed to clarify the exact role of cAMP signaling in the A_{2A} receptor-mediated modulation of behavioral sensitization.

The A_{2A} receptor may also exert its effect on behavioral sensitization through a network-level interaction with D_1 receptors, which are primarily expressed on non-A_{2A} receptor-expressing

striatonigral neurons (of the "direct" pathway). Interestingly, Pollack et al. (1997) showed that "priming" by the dopamine agonist apomorphine leads to the recruitment of D_2 receptor-expressing in concert with D_1 receptor-expressing striatal neurons to produce enhanced behavioral and cellular responses. Similarly, in striatal slices, activation of the D_2 or A_{2A} receptors transynaptically interacts with D_1 receptors to modulate phosphorylation of DARPP-32, an effect that was blocked by tetrodotoxin (Lindskog et al., 1999). These studies illustrate clearly a functional cross-talk between the indirect pathway (coexpressing A_{2A} and D_2 receptors) and the direct pathway (expressing D_1 receptors) at the network level. Furthermore, in striatonigral neurons of the "direct" pathway, the increased expression of mRNAs encoding neuropeptides substance P and dynorphin has been associated with the development of behavioral sensitization in response to repeated L-DOPA (Engber et al., 1991; Herrero et al., 1995; Cenci et al., 1998; Andersson et al., 1999; Pirker et al., 2001), indicating overactivity of the (D_1-expressing) "direct" pathway in L-DOPA-sensitized animals. Our finding that chronic L-DOPA treatment reverses the 6-OHDA-induced reduction in striatal dynorphin mRNA in WT but A_{2A} KO mice supports this notion. It is also consistent with our preliminary finding that D_1 agonist challenge after the chronic L-DOPA treatment schedule used in this study resulted in enhanced contralateral turning (compared with baseline rotational response to D_1 stimulation before L-DOPA treatments) in WT but not A_{2A} KO mice (S. Fredduzzi, M. A. Schwarzschild, and J.-F. Chen, unpublished observations). Thus, A_{2A} receptor modulation of dopaminergic sensitization may involve both D_1 and D_2 dopamine receptors in the "direct" striatonigral and "indirect" striatopallidal pathways, respectively.

Finally, A_{2A} receptors may modulate L-DOPA-induced behavioral sensitization by modulating presynaptic release of several neurotransmitters such as dopamine, glutamate, and GABA, all critically involved in the development of dopamine-associated behavioral sensitization (Pierce and Kalivas, 1997). Although activation of A_1 receptors at presynaptic terminals markedly inhibits the release of a wide-range of neurotransmitters, activation of A_{2A} receptors has been shown to enhance dopamine release in striatum and nucleus accumbens (Okada et al., 1996; Sebastiao and Ribeiro, 1996). This is further supported by the recent demonstration that basal dopamine levels in striatum, measured by microdialysis, were significantly lower in A_{2A} KO mice than that of WT littermates (Dassesse et al., 2001), and is consistent with our findings that depolarization-elicited dopamine release was significantly attenuated in striatal synaptosomes from A_{2A} KO mice (T. Turner, J.-F. Chen, and M. A. Schwarzschild, unpublished observations). Thus, inactivation of A_{2A} receptors may reduce dopamine release resulting in hypo-dopaminergic activity, and in turn, attenuated L-DOPA sensitization.

In addition to dopamine, glutamate and its release from nerve terminals in the striatum, nucleus accumbens, and ventral mesencephalon have been strongly implicated in the different phases of sensitization (for review, see Wolf, 1998). A_{2A} receptors serve a well established excitatory CNS role (Sebastiao and Ribeiro, 1996) attributed to their facilitative effects on glutamate release, which have been demonstrated in striatum (Popoli et al., 1995) as well as cortex (O'Regan et al., 1992) Thus, an attenuation of glutamate release in A_{2A} KO mice may contribute to their phenotype of attenuated L-DOPA-induced sensitization. Alternatively, A_{2A} receptor-enhancement of GABA release particularly from A_{2A} receptor-expressing striatopallidal neurons (Kurokawa et al., 1994; Mayfield et al., 1996) also offers a plausible basis for the reduced L-DOPA sensitization in KO mice. Modulation of striatal acetylcholine (as well as dopamine) release by A_{2A} receptors (Zetterstrom and Fillenz, 1990; Kurokawa et al., 1994, 1996) are also potential targets of A_{2A} receptors involvement.

Therapeutic implications for PD

The lack of persistent L-DOPA-induced behavioral sensitization in A_{2A} KO mice has implications for the development of A_{2A} antagonists as a potential therapeutic intervention for PD and psychiatric disorders associated with dopaminergic system dysfunction. A_{2A} antagonists are being developed as novel therapeutic agents for PD treatment based primarily on their well documented capacity to enhance motor function. In animal models of PD, A_{2A} antagonists alone or in combination with L-DOPA reverse parkinsonian motor deficits in rodents and nonhuman primates (Richardson et al., 1997; Kanda et al., 1998; Grondin et al., 1999). In addition, we have recently demonstrated that A_{2A} antagonists attenuate dopaminergic neurotoxicity in the MPTP model of PD in mice, raising the possibility that A_{2A} antagonists may offer neuroprotective as well as symptomatic benefits in PD treatment (Chen et al., 2001). The present findings suggest an additional potential benefit of A_{2A} receptor inactivation as adjunctive therapy with L-DOPA in PD. Persistent rotational sensitization and grooming sensitization induced by repeated L-DOPA administration was dependent after the presence of the A_{2A} receptor, and thus A_{2A} antagonists may attenuate the maladaptive dyskinetic responses to chronic L-DOPA treatment in PD.

Together, these multiple potential therapeutic benefits of A_{2A} antagonists (motor enhancement, neuroprotection against dopaminergic toxicity, and the prevention of dyskinesia), coupled with their low risk of CNS side effects (given the relatively discrete striatal expression of the A_{2A} receptor) should greatly encourage their development as a promising treatment for PD. The first clinical trials to evaluate a specific A_{2A} antagonist are now targeting advanced PD patients suffering from dyskinesias and related motor fluctuations. This strategy is supported the preclinical evidence that A_{2A} receptor blockade may enhance motor function without inducing or exacerbating dyskinesias (Kanda et al., 1998; Grondin et al., 1999). However, the new findings suggesting additional benefits of attenuated dyskinesia development and disease progression in PD provide a strong rationale for considering the use of A_{2A} antagonists early in the course of the disease.

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