Rescue of Locomotor Impairment in Dopamine D2 Receptor-Deficient Mice by an Adenosine A2A Receptor Antagonist

Shiro Aoyama,¹,² Hiroshi Kase,² and Emiliana Borrelli¹

¹Institut de Génétique et de Biologie Moléculaire et Cellulaire, BP163, 67404 Illkirch Cedex, C.U. de Strasbourg, France, and ²Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., Nagažumi-cho Shizuoka, 411-8731 Japan

In Parkinson’s disease a degeneration of dopaminergic neurons of the nigrostriatal pathway is observed. Loss of dopaminergic regulation of striatal neuron activity results in altered motor functions. Adenosine A2A (A2AR) and dopamine D2 (D2R) receptors are colocalized in striatal medium spiny neurons. It has been proposed that adenosine binding to A2AR lowers the affinity of dopamine for D2R, thus modulating the function of this receptor. Absence of D2R in knockout mice (D2R⁻/⁻) results in impaired locomotion and coordinated movements. This indicates that absence of dopamine in Parkinson’s disease might principally affect D2R-mediated effects with regard to locomotor functions. A2AR-selective antagonists have been demonstrated to have anti-parkinsonian activities in various models of Parkinson’s disease in rodents and nonhuman primates. In this article, D2R⁻/⁻ mice were used to explore the possibility that an A2AR antagonist might reestablish their motor impairment. Interestingly, blockade of A2AR rescues the behavioral parameters altered in D2R⁻/⁻ mice. In addition, the level of expression of enkephalin and substance P, which were altered in D2R⁻/⁻, were also reestablished to normal levels after A2AR antagonist treatment. These results show that A2AR and D2R have antagonistic and independent activities in controlling neuronal and motor functions in the basal ganglia. They also provide evidence that selective A2AR antagonists can exhibit their anti-parkinsonian activities through a nondopaminergic mechanism.

Key words: adenosine A2 receptor; dopamine D2 receptor; striatum; KW-6002; Parkinson’s disease; knockout mice

Parkinson’s disease affects the major component of the basal ganglia, the striatum, 90% of which is composed of GABA-releasing medium-sized spiny neurons. These neurons receive dopaminergic afferent fibers from the substantia nigra as well as massive glutamatergic cortical afferences. They generate the striatopallidal, or indirect, and striatonigral, or direct, pathways. The direct pathway relieves inhibition of thalamocortical neurons and facilitates desired movements, whereas the indirect pathway causes inhibition of these neurons and inhibits undesired movements. Dopamine depletion in Parkinson’s disease causes an increased function of the striatopallidal neurons, leading to an unbalanced activity of these pathways (DeLong, 1990; Obeso et al., 1997).

A recently developed adenosine A2AR selective antagonist, (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (KW-6002) exhibits anti-parkinsonian activities in rodent as well as in nonhuman primate models of the disease (Shimada et al., 1997, Shiozaki et al., 1999, Kanda et al., 1998). Adenosine A2A receptors (A2ARs) are localized at high density in the basal ganglia (Jarvis and Williams, 1989), where they seem to influence motor behavior (Ferré et al., 1991a; Nikodijevic et al., 1991; Richardson et al., 1997). In particular, A2ARs are predominately expressed in striatopallidal neurons that also express dopamine D2 receptors (D2Rs) (Fink et al., 1992). The mechanism by which adenosine affects motor functions has been thought to be attributable to modulation of dopamine-mediated signaling. It is presently unclear whether adenosine has an independent role in the control of locomotion. Interactions between A2AR and D2R activities have been shown at the plasma membrane and intracellular levels. A2AR stimulation affects the affinity of dopamine for D2R (Ferré et al., 1991b), counteracts D2R-mediated Ca²⁺ influx (Yang et al., 1995), and abolishes the D2R-mediated tonic inhibition of c-fos expression (Boegman and Vincent, 1996). Thus, it has been proposed that A2AR–D2R interactions may constitute the main molecular mechanism underlying many of the observed effects of adenosine agonists and antagonists (Ferré et al., 1997). However, increasing evidence suggests that A2ARs can operate independently from D2Rs by modulating GABA release and recurrent feedback inhibition of striatal neurons (Kurokawa et al., 1994; Mori et al., 1996). This illustrates a potential key function of adenosine in the control of movements (Richardson et al., 1997).

D2R knockout mice (D2R⁻/-) (Baik et al., 1995; Kelly et al., 1998; Jung et al., 1999) thus provide a tool to evaluate the role of A2AR in motor control as related to dopamine receptor function. D2R⁻/- mice present a locomotor phenotype with analogies to Parkinson’s disease (Baik et al., 1995). In these mice we evaluated the effect of KW-6002 treatments on the rescue of their locomotor abnormalities as well as on striatal neuropeptide gene expression as an indicator of D2R⁻/- mice neuronal activity. The results indicate that blockade of A2AR is able to rescue the locomotor deficits of D2R⁻/- mice probably by modulating striatal projection neuron activities, even in the absence of D2R. These results show a dopamine-independent role for adenosine in the control of motor functions through A2AR in the striatum. They also illustrate a novel non-D2R-dependent mechanism for the anti-parkinsonian activities of selective A2AR antagonists.

MATERIALS AND METHODS

Mice. D2R⁻/- mice (Baik et al., 1995) had a mixed 129SV/C57BL/6 genetic background, with a 75% contribution of C57BL/6. Genotypes of mice were confirmed by genomic Southern hybridization analyses. Mice were group-caged and maintained in a light-controlled room (12 hr light/dark cycle) under standard animal housing conditions. Experiments were performed in conformity with the French publication on animal experimentation (no. 87–848).

Drug treatment and behavioral tests. KW-6002 was synthesized at the Medicinal Chemistry Department of the Pharmaceutical Research Institute (Shizuoka, Japan). The behavioral experiments were performed between 2 and 8 P.M. Wild-type (WT) and D2R⁻/- littermates were administered 2.5 mg/kg KW-6002 (suspension in 0.3% Tween 80, 10 ml/kg, p.o.) or vehicle (0.3% Tween 80). Locomotor activity was measured in a Plexiglas cage (30 × 50 × 15 cm), the floor of which was partitioned into 12 squares of equal surface area (Janssen et al., 1960). Mice were

Copyright © 2000 Society for Neuroscience 0270-6474/00/205848-05$15.00/0

Received April 5, 2000; revised May 8, 2000; accepted May 9, 2000.

This work was supported by grants from Kyowa Hakko Kogyo, Co., Ltd., Centre National de la Recherche Scientifique, Institut National de la Sante et de la Recherche Médicale, MILDT, and ARC to E.B. We thank M. Kobayashi and N. Foulkes for discussions.

Correspondence should be addressed to Dr. Emiliana Borrelli, Institut de Génétique et de Biologie Moléculaire et Cellulaire, BP163, 67404 Illkirch Cedex, C.U. de Strasbourg, France. E-mail: eb@ibgmc.u-strassbg.fr.

Copyright © 2000 Society for Neuroscience 0270-6474(00)205848-05$15.00/0
placed in the cages and left 1 hr for habituation, then administered the drug (n = 6, WT; n = 7, D2R−/−) or vehicle (n = 6, WT; n = 8, D2R−/−) and returned to the cage. The behavior of the mice was recorded by video camera for each 15 min during a 120 min session. For the rotated test (Wolfgramm et al., 1990), each mouse was placed on a 3.5-cm-diameter rod covered with rubber 2.5 hr after drug (n = 6, WT; n = 9, D2R−/−) or vehicle (n = 12, WT; n = 9, D2R−/−) treatment. Animals were left for 1 min on the rod to habituate. The rod position was changed randomly and the behavior of mice was scored for a maximum of 3 min. The best performance among three trials was used for analysis. For the ring test (Pertwee, 1972), each mouse was placed on a 5.5-cm-diameter ring attached to a stand 16 cm above the base. 2.5 hr after treatment with drug (n = 6, WT; n = 9, D2R−/−) or vehicle (n = 18, WT; n = 9, D2R−/−) treatment, and the behavior was video-recorded for 5 min. The length of the period without any voluntary movements was measured.

**RESULTS**

A2AR and D2R are coexpressed in striatal medium spiny neurons. Therefore, we investigated whether absence of D2R in mutant mice has some influence on the expression and the pharmacological characteristics of A2AR.

No alterations in the distribution and level of expression of the

Figure 2. Expression of adenosine A2AR mRNA in striatum of WT, heterozygote, and D2R−/− mice. A, B. Adenosine A2AR in situ hybridizations on coronal sections from WT and D2R−/− mice (KO). C. Northern blot analysis of D2R expression in striata from WT, heterozygote D2R+/− (Hetero), and D2R−/− (KO) mice. Values are presented as percentage of WT control.

Figure 3. Effect of KW-6002 treatment on striatal neuropeptide expressions. D2R−/− and WT mice were treated with KW-6002 (2.5 mg/kg) or vehicle. Mice were killed 3 hr after treatment. Neuropeptide mRNA expression levels in each treatment are expressed as percentage of control (WT treated with vehicle) ± SEM from 4–11 individuals in each group. **, p < 0.01 versus wild type with vehicle treatment. †, p < 0.05; †††, p < 0.001 versus D2R−/− mice with vehicle treatment by Student’s t test.
A2AR mRNA in D2R−/− mice were found as compared with WT littermates (Fig. 1A, B).

To pharmacologically characterize A2ARs in D2R−/− mice, ligand binding experiments were performed using the A2AR agonist [3H]CGS21680 on striatal membranes. No significant differences in the affinity and density of [3H]CGS21680 binding sites were found (WT mice: Kd 0.5 ± 0.6 nM and Bmax 117.5 ± 141.3 fmol/mg protein; D2R−/− mice: Kd 7.5 ± 0.5 nM and Bmax 123.3 ± 37.3 fmol/mg protein) (Fig. 2). Thus, neither the expression nor the pharmacological properties of A2ARs are affected by the loss of D2R expression in vivo.

**KW-6002 is able to reverse altered neuropeptide levels in D2R−/− mice**

D2R−/− mice exhibit altered gene expression of striatal neuropeptides, such as enkephalin and substance P (Baik et al., 1995), which reflect alteration of the activities in the striatopallidal and striatonigral neurons, respectively. Enkephalin is coexpressed with D2R and A2ARs in the striatopallidal neurons, whereas SP and dynorphin colocalize with D1R in striatonigral neurons (Gerfen et al., 1990).

Comparative studies on the expression of these genes were performed on total RNA extracted from the striatum of D2R−/− and WT mice after treatment with KW-6002 or saline. In agreement with previous studies (Baik et al., 1995), a 32% increase in enkephalin mRNA expression and a 23% decrease in SP mRNA were observed in D2R−/− mice as compared with WT animals (Fig. 3). Interestingly, KW-6002 administration (2.5 mg/kg) in the mutants decreased enkephalin expression (93% of WT, n = 5) (Fig. 3) and increased SP expression (118% of WT, n = 5) (Fig. 3) to normal WT levels.

Thus KW-6002 is able to reverse the altered neuropeptide's expression caused by the loss of D2R signaling, indicating that the blockade of adenosine A2AR normalizes the unbalanced neuronal activities of striatal output pathways.

**KW-6002 rescues motor activity in D2R−/− mice**

D2R−/− mice have impaired locomotion characterized by a reduction of spontaneous movements and stereotypes. If normalization of imbalanced activity of striatal neurons on KW-6002 treatment occurs in D2R−/− mice, this should also lead to an amelioration of their motor impairment. Thus, behavioral tests originally used to characterize D2R−/− mice (open field, rotarod, and ring test) were performed.

Animals from both genotypes were habituated to the open field before being orally administered KW-6002 or vehicle (Fig. 4A). Mice were then administered either KW-6002 or saline, and the number of crossed squares and rearings were counted for the next 2 hr (Table 1). KW-6002 produced a similar significant increase in ambulation in both genotypes (WT, p = 0.0005; D2R−/−, p = 0.0017) and rearings (WT, p = 0.0001; D2R−/−, p = 0.004) (Fig. 4A, B, Table 1). These effects lasted during the 2 hr of the test. A two-way ANOVA grouped on genotype and treatment showed a significant interaction between genotype and treatment (ambulation, F(1,23) = 25.3; rearing, F(1,23) = 80.9). However, a similar percentage increase in ambulation and rearings was observed in the two genotypes with respect to their basal level of locomotion (Table 1). These results indicate that KW-6002 induces hyperactivity in the WT as well as in the knockouts, demonstrating a D2R-independent function for the A2AR. Furthermore, this compound is able to completely rescue the locomotor impairment of D2R−/− mice to saline-treated WT levels.

Coordination of movements was tested using the rotarod apparatus. D2R−/− mice are not able to correctly perform this test (Baik et al., 1995), and they spent significantly shorter time on the rotating rod than WT littermates (p = 0.017) (Fig. 5A). Importantly, KW-6002 restored D2R−/− mice performance and prolonged the time spent on the rod in both genotypes (p = 0.0005 for WT, p = 0.0075 for D2R−/−) (Fig. 5A). ANOVA grouped on genotype and treatment showed that there was no genotype–treatment interaction (F(1,32) = 0.8, p = 0.38).

Spontaneous movements that were quantified using the ring test showed fewer voluntary movements in D2R−/− than in WT mice (Fig. 5B) (p < 0.0001). KW-6002 increased voluntary movements in both genotypes, shortening the time spent being immobile (Fig. 5B) (p = 0.0021 for WT, p < 0.0001 for D2R−/−). Genotype–treatment interaction was found (F(1,38) = 18.6). Taken together, these results indicate that KW-6002 can rescue the movement impairment elicited by ablation of D2Rs.

**DISCUSSION**

D2R−/− mice present an impairment of locomotor functions, indicating a preponderant role for D2R in the control of locomotion (Baik et al., 1995; Kelly et al., 1998; Jung et al., 1999). D2Rs are...
colocalized with A2ARs in the striatal medium spiny neurons. A2AR-mediated signaling may be involved in the control of motor functions (Ferré et al., 1992). New highly selective A2AR antagonists have been demonstrated to exert anti-parkinsonian activities in MPTP-treated rodents and primates (Kanda et al., 1998; Shiozaki et al., 1999). D2Rs and A2ARs have opposite effects on the cAMP transduction pathway, which might have contrasting effects on biochemical and behavioral parameters (Ferré et al., 1997). We thus investigated whether blockade of the A2AR in D2R−/− mice might reestablish some of the motor and biochemical deficits found in these mice, via the regulation of neuronal activities in striatal neurons.

mRNA and binding properties of the A2AR are unmodified in D2R−/− mice (Figs. 1, 2). Thus, the absence of D2R does not result in a compensatory modification of the expression level and binding characteristics of A2AR. This is consistent with previous reports showing that the number of A2AR binding sites is not altered by deficits in dopaminergic transmission (Morelli et al., 1994; Przedborski et al., 1995) as well as in Parkinson’s disease (Martinez-Mir et al., 1991). This implies that there might not be direct membrane interactions between A2AR and D2Rs, as has been postulated (Ferré et al., 1992, 1997).

It has been demonstrated previously that dopamine depletion, D2R blockade by antagonists, and D2R gene knockout results in an increase in the expression of the preproenkephalin gene (Augood et al., 1989; Baik et al., 1995; Richardson et al., 1997). Conversely, the knockowut of the A2AR (Ledent et al., 1997) as well as antagonist blockade (Richardson et al., 1997) of this receptor leads to a decrease of preproenkephalin expression in the striatum. In agreement with this, our results demonstrate that KW-6002 is able to reverse the alterations observed in enkephalin and SP expression caused by the absence of D2R. These results clearly establish a relevant and antagonistic physiological control exerted by A2AR and D2R on enkephalin expression, which is coexpressed in the same neurons. These results support an independent function in vivo of these two receptors, which is compatible with electrophysiological data showing that presynaptic A2ARs activate striatopallidal neurons by counteracting striatopallidal GABAergic neurotransmission (Kurokawa et al., 1994; Mori et al., 1996).

Interestingly, in D2R−/− and A2AR−/− mice, a reduction of SP expression has been observed (Baik et al., 1995; Ledent et al., 1997) despite the proposed localization of these receptors and SP in different neurons (Le Moine et al., 1991; Schiffmann et al., 1991). Immunohistochemical evidence has shown that the spiny neurons of the direct and indirect pathways are synaptically interconnected (Arnon et al., 1986; Yung et al., 1996). In D2R−/− mice, SP reduction might be dependent on the inhibition of striatonigral neurons by overactive striatopallidal collaterals. Blockade of A2ARs on these neurons, by restoring striatopallidal activity, reduces the inhibition on striatonigral neurons resulting in an increase and a normalization of SP expression in mutant mice. This might be the mechanism responsible for the upregulation of SP mRNA expression in D2R−/− mice, on administration of KW-6002. Alternatively, this effect might be mediated by cholinergic interneurons also expressing A2AR (Richardson et al., 2000). Indeed, A2AR antagonists reduce ACh release (Kurokawa et al., 1996), and the blockade of muscarinic receptors increases SP expression (Lucas and Harlan, 1995). Thus KW-6002 treatment might suppress ACh release in the striatum, and then the decreased ACh tone results in an increase of SP mRNA expression in the mutants. The locomotor stimulant effect of KW-6002 on WT animals seems to be higher than in the D2R−/− group, and a genotype–treatment interaction is revealed by the ANOVA analysis. However, this result might be dependent on the habituation of both groups of animals to the open field because the treatment was given when the animals were at rest. Thus, the basal activity of the WT group, as compared with the D2R group, might have been underestimated. Interestingly, a genotype–treatment interaction has not been found in the rotarod test. This test evaluates higher integration of motor function and body balance, such as combined movement of forelimbs and hindlimbs on the rod. We might speculate that the extent of the contribution of adenosine to some behavior as related to locomotor functions is different.

A balance of the direct and indirect pathways is required for the accomplishment of normal movements (Gerfen, 1992). Movement impairments in Parkinson’s disease and in D2R−/− mice are the result of an imbalance in the activity of these pathways caused by the reduction of dopamine in the first case and of D2R-mediated signaling in the second. Behavioral studies on D2R−/− mice treated with KW-6002 revealed that this selective A2AR antagonist is able to rescue the motor impairment observed in these mice. This suggests that blockade of A2A receptor might reduce the imbalance activity of striatal neurons. Importantly, adenosine KW-6002 restores the movement impairment in mutant mice in the absence of D2 receptors, indicating that its activity is independent of D2-mediated mechanisms. In normal conditions, where the nigrostriatal projections and D2Rs functions are preserved, independent A2AR and D2R activities as well as A2AR/D2R intramembrane or intracellular interactions might occur in striatopallidal neurons. However, in conditions where dopamine or D2R activity is lowered or absent (e.g., in Parkinson’s disease patients, MPTP-exposed monkeys, and D2R knockout mice), adenosine through A2AR may still elicit its proper physiological effects independently from the antagonistic interaction with dopamine-mediated signaling. This has important relevance in developing novel therapies for Parkinson’s disease.

In conclusion, our study shows that KW-6002 treatment of D2R−/− mice reestablishes their altered striatal gene expression and locomotor behavior. Blockade of A2ARs might decrease the neuronal activity of the striatopallidal ACh-mediated pathway in the absence of D2R-mediated signaling. This strongly suggests that A2AR and D2R have antagonistic but independent functions in the striatum. These results also show that adenosine should be
considered as one of the determinants in the control of striatal neuronal activities relevant to motor functions.

**REFERENCES**


Jung M-Y, Skryabin BV, Arai M, Abbondanzo S, Fu D, Brosius J, Robakis NK, Pertwee RG (1992) The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci 15:133–139.


Aoyama et al. • A2AR Antagonist Rescues Locomotion in D2R−/− Mice