Adenosine A_{2A} Receptor Antagonists Exert Motor and Neuroprotective Effects by Distinct Cellular Mechanisms

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Objective: To investigate whether the motor and neuroprotective effects of adenosine A_{2A} receptor ($A_{2A}R$) antagonists are mediated by distinct cell types in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease.
Methods: We used the forebrain $A_{2A}R$ knock-out mice coupled with flow cytometric analyses and intracerebroventricular injection to determine the contribution of $A_{2A}Rs$ in forebrain neurons and glial cells to $A_{2A}R$ antagonist-mediated motor and neuroprotective effects.
Results: The selective deletion of $A_{2A}Rs$ in forebrain neurons abolished the motor stimulant effects of the $A_{2A}R$ antagonist KW-6002 but did not affect acute MPTP neurotoxicity. Intracerebroventricular administration of KW-6002 into forebrain $A_{2A}R$ knock-out mice reinstated protection against acute MPTP-induced dopaminergic neurotoxicity and attenuated MPTP-induced striatal microglial and astroglial activation.
Interpretation: $A_{2A}R$ activity in forebrain neurons is critical to the control of motor activity, whereas brain cells other than forebrain neurons (likely glial cells) are important components for protection against acute MPTP toxicity.

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In recent years, adenosine A_{2A} receptors ($A_{2A}Rs$) have emerged as a leading nondopaminergic therapeutic target in Parkinson's disease (PD).[1–3] This excitement is primarily due to two lines of recent experimental and clinical evidence. First, selective $A_{2A}R$ antagonists such as KW-6002 have been shown to stimulate motor activity, alone or in combination with L-dopa, to provide symptomatic relief in PD as evidenced in rodents,[4,5] nonhuman primates,[6,7] and recent clinical studies.[8,9] Second, epidemiological studies show an inverse relation between caffeine consumption and the risk for development of PD,[10,11] and animal studies show that $A_{2A}Rs$ contribute to the degeneration of nigrostriatal dopaminergic neurons,[12,13] raising the prospect that $A_{2A}R$ antagonists may also represent a neuroprotective strategy for PD. Thus, the use of $A_{2A}R$ antagonists could constitute a significant improvement in PD therapy compared with the current L-dopa monotherapy, which does not slow down dopaminergic neurodegeneration.[14]

The dual motor/protective benefit of $A_{2A}R$ antagonists for PD patients raises an important question: Is the motor stimulation and neuroprotection exerted by $A_{2A}R$ antagonists mediated by distinct or common cellular mechanisms? Although the antagonistic interaction between $A_{2A}R$ and D_2 dopamine receptors in striatopallidal neurons is considered the molecular basis for $A_{2A}R$-mediated motor stimulation,[15] the mechanism by which $A_{2A}R$ inactivation attenuates dopaminergic degeneration is largely unknown. Given the predominant expression of $A_{2A}Rs$ in the striatal neurons, $A_{2A}Rs$ in forebrain neurons are believed to be responsible not only for motor control but also for the neuroprotection afforded by $A_{2A}R$ antagonists. In addition, mechanisms involving various cellular and molecular elements (including dopaminergic neurons, microglial cells, vesicular monoamine transporter, and monoamine oxidase-B) have also been suggested.[16] The dissection of the cellular mechanism underlying motor stimulation and neuroprotection by $A_{2A}R$ antagonists

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may provide a basis for achieving the dual benefits by targeting A_{2A}Rs in distinct cells.

The key to addressing this important issue is to distinguish the contributions of A_{2A}Rs in different cell types to motor stimulation and neuroprotection in PD models. However, current pharmacological and global genetic knock-out (KO) approaches to A_{2A}Rs are not adequate to discern the specific actions of A_{2A}Rs in different cell types. To circumvent this limitation, we developed forebrain neuron-specific A_{2A}R KO mice (fb-A_{2A}R KO) by crossing "floxed" A_{2A}R mice with the calmodulin-dependent protein kinase II α subunit (CaMKIIα)-Cre transgenic line.[17] Using this novel forebrain A_{2A}R KO line together with pharmacological approaches, we provide the first direct evidence that A_{2A}R antagonists exert their motor and neuroprotective benefits by targeting forebrain neurons and brain cells other than forebrain neurons (such as glial cells), respectively, in the MPTP model of PD.

Materials and Methods

Transgenic Mice

The animals were handled in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. The generation and characterization of the global A_{2A}R KO mice (gb-A_{2A}R KO) of congenic C57BL/6 genetic background (N10) have been described previously.[18] The generation and genotyping of the forebrain A_{2A}R KO mice (fb-A_{2A}R KO) of near congenic C57BL/6 background (N6) have also been described recently.[17] Both Cre(−)$A_{2A}R^{flox+/+}$ and Cre(+)$A_{2A}R^{flox-/-}$ were used as wild-type (WT) control (referred to as fb-WT) to compare with fb-A_{2A}R KO [Cre(+)$A_{2A}R^{flox+/+}$]. In pilot studies, we found that both WT control animals [Cre(−)$A_{2A}R^{flox+/+}$ and Cre(+)$A_{2A}R^{flox-/-}$] produce similar behavioral (locomotor) and neurochemical (striatal dopamine contents) effects. All animals in this study were 90 days or older due to the CaMKIIα-Cre–mediated A_{2A}R deletion time course.

Locomotor Activity and Drugs

Horizontal locomotor activity was assessed in standard activity cages as described previously.[12,18] All mice were habituated to the testing environment, and basal locomotion was recorded before and after drug treatments. Mice were monitored during the light or dark phase of the light/dark cycle to obtain low and high baseline locomotor activity when studying A_{2A}R antagonists and A_{2A}R agonists. KW-6002[19] was dissolved in vehicle (15% DMSO, 15% castor oil, and 70%H_2O) and CGS21680 (Tocris, Ellisville, MO) was dissolved in saline.

In Situ Hybridization

Postfixed brain sections were hybridized with 0.4nM ^{35}S-oligonucleotide probe (approximately 1.5×10^6cpm per 80μl per slide) at 37°C overnight and washed at high strength as described previously.[20] The A_{2A}R oligonucleotide probe was mapped at 51 to 95bp of the A_{2A}R complementary DNA sequence (NM_009630).

Membrane Binding Assay

Total membranes or Percoll-purified synaptosomal membrane preparations from frontal cerebral cortex, striatum, or hippocampus were prepared as described previously.[21] Membrane preparations were incubated with ^{3}H-SCH58261 (specific activity of 77Ci/mmol; a gift from Dr Ennio Ongini, Shering-Plough, Italy) for 1 hour, as described previously.[21] Xanthine amine conger (1μM) was used to assess nonspecific binding.

Western Blot Analysis

Total membrane preparations of striatum were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred, and incubated with a mouse anti-A_{2A}R antibody (1:1,000; Upstate Biotechnology, Lake Placid, NY) as described previously. The membranes were developed with enhanced chemifluorescence and quantification achieved with the Quantity One System (Bio-Rad, Richmond, CA).[21]

Immunohistochemistry

Perfused brain were sectioned coronally (30μm), and immunohistochemistry was performed as described previously.[18,22] In brief, the sections were incubated with an anti–mouse A_{2A}R (1:500; Upstate Biotechnology) and developed with peroxidase-diaminobenzidine tetrahydrochloride solution (Vector Laboratories, Burlingame, CA). Sections from midbrain were stained with anti–mouse tyrosine hydroxylase (1:200; Chemicon International, Temecula, CA). The tyrosine hydroxylase–positive nigral cells were counted manually by an observer blind to the treatment. The average cell counts of two sections from each animal were used to calculate cell numbers. To further evaluate the colocalization of A_{2A}Rs in neuronal and glial cells in fb-A_{2A}R-KO, we treated the mice with MPTP (40mg/kg intraperitoneally [ip]) 2 days later, brains were sectioned through striatum, and we incubated the section with mouse anti-A_{2A}R (1:500 dilution; Upstate Biotechnology) in combination with either rabbit anti–β-tubulin III (1:1,000; Covance, Princeton, New Jersey), anti-CD11b (1:100; Serotec, Bicester, United Kingdom), or anti–glial fibrillary acidic protein (GFAP; 1:500; Dako, Carpinteria, CA), followed by incubation with an anti–mouse IgG conjugated with Alexa Fluor 488 and an anti–rabbit IgG conjugated with Alexa Fluor 594 (1:200; Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst 33342 (2μg/ml). The brain sections were analyzed in a laser-scanning confocal microscope (LSM 510 META; Zeiss, Thornwood, NY).

Fluorescent Activated Cell Sorting/Flow Cytometry Analysis

Brain tissue of different regions was cut into small pieces, treated briefly with 0.025% trypsin-EDTA (Invitrogen, La Jolla, CA) at room temperature, and filtered through a 70μm cell constrainer (BD Biosciences, San Jose, CA) to obtain single-cell suspension. The cells were then incubated with anti–mouse β-tubulin III (1:50; Sigma-Aldrich, St. Louis, MO), Fluor 488–conjugated anti–mouse GFAP (1:

100; Molecular Probes) or Phycoerythrin-conjugated anti–mouse CD11b (1:100; BD Biosciences). The cells were permeabilized with 0.5% saponin for 15 minutes on ice before labeling with the anti–β-tubulin III and anti-GFAP antibodies, followed by an extended wash procedure (4 washes of 10 minutes each) to reduce the background staining. The cells were either separated using a fluorescent activated cell sorter (MoFlo; Dako) for genotyping and membrane binding assay or scanned for fluorescence intensity on a flow cytometry scan (BD Biosciences). The genomic DNA was extracted from the sorted cells to detect Cre-mediated $A_{2A}R$ gene deletion by polymerase chain reaction. For the $A_{2A}R$ binding study, about one million sorted $CD11b^+$ cells isolated from the cerebral cortex and hippocampus of three to four fb-$A_{2A}R$ KO or fb-WT mice were pooled to determine the ^3H-SCH58261 binding in these cells.

MPTP Treatment Paradigms and Catecholamine Measurements

Three MPTP treatment paradigms were used in this study. First is the multiple-injection paradigm: Mice received three intraperitoneal injections of 20mg/kg MPTP at 2-hour intervals.[12] Second is the single-injection paradigm: A single injection paradigm of 40mg/kg MPTP was used to further validate the three-injection paradigm and to accommodate the intracerebroventricular injection paradigm of the $A_{2A}R$ antagonist KW-6002. KW-6002 (10μg/2μl/brain) was injected intracerebroventricularly (ICV) using a stereotaxic apparatus (anterior −1.0mm, depth −2.0mm, and lateral −0.5mm, relative to Bregma). Ten minutes later, the mice were administered a single injection (IP) of 40mg/kg MPTP. Third is the subchronic treatment paradigm: Daily injection of MPTP (30mg/kg IP) for 5 days as described previously[5] to deplete dopamine. Thirty minutes after the last injection of MPTP (day 5), the mice were treated with KW-6002 (3mg/kg IP) and their locomotor activities were recorded for 120 minutes. MPTP-induced neurodegeneration was assessed 7 days after the last injection. Striatal dopamine levels were assessed by standard reverse-phase high-pressure liquid chromatography with electrochemical detection.[12]

Statistical Analysis

Statistical comparisons were performed by unpaired Student's *t* test or one-way analysis of variance followed by Bonferroni post hoc comparisons. Otherwise, nonparametric Mann–Whitney *U* test or Kruskal–Wallis H test was used. For behavioral analysis, we performed two-way analysis of variance for repeated measurements followed by Bonferroni post hoc comparison for the effects of genotype, drug treatment, and their interaction.

Results

Genetic and Biochemical Characterization of Forebrain Adenosine A_{2A} Receptor Knock-out Mice

The deletion of the $A_{2A}R$ gene mediated by CaMKIIα-Cre was detected only in Cre(+) mice specifically in forebrain regions (Fig 1A). Analyses by binding assays (see Fig 1B), in situ hybridization (data not shown), and Western blotting (see Fig 1C) show that the $A_{2A}R$ mRNA, and protein levels were abolished to the background levels observed in the gb-$A_{2A}R$ KO mice. To characterize the specificity of $A_{2A}R$ gene deletion at the cellular level, we isolated neurons (β-tubulin III^+), microglia ($CD11b^+$), and astrocytes ($GFAP^+$) from fb-$A_{2A}R$ KO and fb-WT mice ($Cre(-)flox^{+/+}$) by fluorescent activated cell sorting. The polymerase chain reaction and binding analyses of the sorted cells indicates that in the fb-$A_{2A}R$ KO mice, the deletion of $A_{2A}R$ gene (at genomic DNA and protein levels) is neuron specific (Figs 2A, B) because neither the $A_{2A}R$ gene nor the $A_{2A}R$ protein density on $CD11b^+$ (ie, microglial) cells are affected as indicated by polymerase chain reaction (see Fig 2A) and binding (see Fig 2B) assays. Furthermore, double immunohistochemistry showed that the induction of $A_{2A}R$ expression by MPTP treatment was colocalized with the expression of astroglial (ie, GFAP+) and microglial markers (ie, $CD11b^+$) in striatum of fb-$A_{2A}R$ KO (see Fig 2C) after the acute MPTP treatment. As expected, no $A_{2A}R$ immunoreactivity was detected in striatal neurons (ie, tubulin-III) of fb-$A_{2A}R$ KO mice (see Fig 2C). Together, these data demonstrate that the newly developed fb-$A_{2A}R$ KO line depleted $A_{2A}R$ messenger RNA and protein in forebrain neurons to levels similar to those of the gb-$A_{2A}R$ KO mice, but $A_{2A}R$ gene and protein density in glial cells remain intact in fb-$A_{2A}R$ KO mice.

Selective Deletion of Adenosine A_{2A} Receptors in Forebrain Neurons Abolishes the Motor Effects of Adenosine A_{2A} Receptor Antagonists and Agonists

We evaluated the effect of the genetic deletion of $A_{2A}Rs$ in forebrain neurons on the $A_{2A}R$ modulation of motor activity. The selective $A_{2A}R$ agonist CGS21680 (0.05mg/kg IP) produced significant motor depression in fb-WT mice that lasted for more than 3 hours (Fig 3A). This motor depressant effect was partially attenuated in fb-$A_{2A}R$ KO mice (see Fig 3A). Next, we assessed the motor stimulant effect of the selective $A_{2A}R$ antagonist KW-6002 and of the nonselective antagonist caffeine in fb-$A_{2A}R$ KO mice. KW-6002 (3mg/kg) produced a strong motor stimulation in fb-WT mice, which was abolished in fb-$A_{2A}R$ KO mice (see Fig 3B). The motor stimulant effect induced by caffeine (10mg/kg) was also largely attenuated in fb-$A_{2A}R$ KO mice (see Fig 3C). Thus, $A_{2A}Rs$ in forebrain neurons are critical for the $A_{2A}R$-mediated motor effects. We next investigated the $A_{2A}R$-mediated motor effects in dopamine-depleted conditions by testing the effect of KW-6002 after a subchronic MPTP treatment paradigm. The locomotor activity was reduced by this subchronic treatment to less then 10% of baseline (before the MPTP treatment). On the last day of treatment, KW-6002 (3mg/kg IP) administered 30 minutes after the last MPTP injection significantly increased

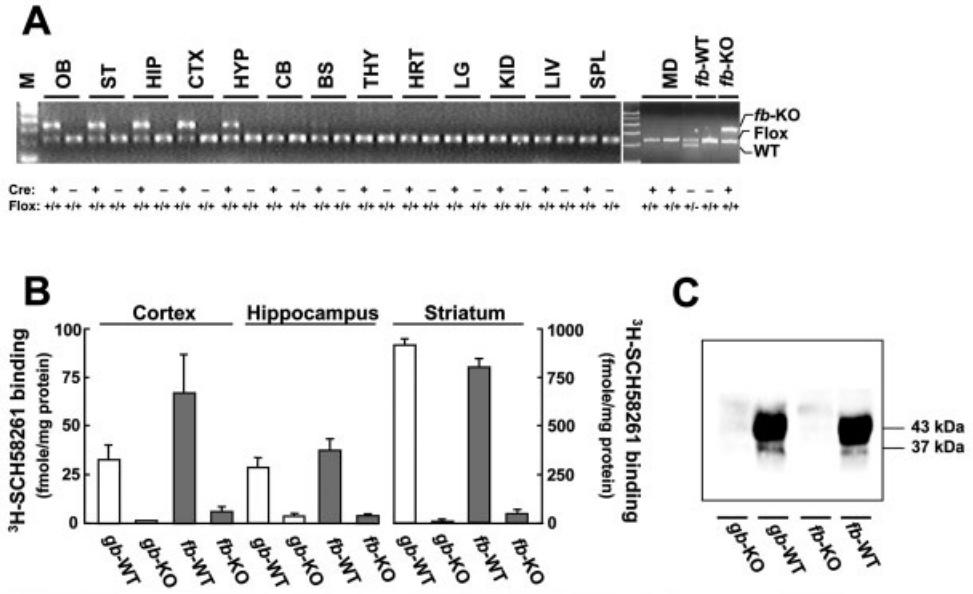

Fig 1. Characterization of the forebrain adenosine A_{2A} receptor ($A_{2A}R$) knock-out (KO) mice. Homozygous "floxed" ($A_{2A}R^{flox+/+}$) mice (F5 generation in a mixed 129-Steel and C57BL/6 genetic background) were crossbred with calmodulin-dependent protein kinase II α subunit (CaMKIIα)-Cre transgenic mice (L7ag13, in a C57BL/6 background) expressing the Cre recombinase under the control of the CaMKIIα gene promoter in forebrain neurons,[37] to generate heterozygous fb-$A_{2A}R$ KO mice [CaMKIIα-Cre(+)$A_{2A}R^{flox+/-}$]. (A) Forebrain-specific, Cre-mediated DNA recombination, as detected by genomic polymerase chain reaction (PCR) analysis, in the mouse tissues from eight brain regions and six peripheral organs that were dissected from fb-$A_{2A}R$ KO mice [Cre(+)$A_{2A}R^{flox+/+}$] or fb-$A_{2A}R$ wild-type (WT) littermates [Cre(−)$A_{2A}R^{flox+/+}$] and genomic DNA were isolated. Cre-mediated $A_{2A}R$ gene deletion ("fb-KO" band) was detected only in the fb-$A_{2A}R$ KO mice but not in fb-WT littermates. Furthermore, Cre-mediated $A_{2A}R$ gene deletion was detected specifically in forebrain regions, including olfactory bulb (OB), striatum (ST), hippocampus (HIP), cerebral cortex (CTX) and hypothalamus (HYP), but not in the cerebellum (CB), brainstem (BS), midbrain (MD; left panel), or in the six peripheral organs, including thymus (THY), heart (HRT), lung (LG), kidney (KID), liver (LIV), and spleen (SPL). (B) Quantitative analysis of ^{3}H-SCH58261 binding densities in whole-membrane preparations of the cerebral cortex, hippocampus, and striatum from gb-$A_{2A}R$ KO mice, fb-$A_{2A}R$ KO mice, and their corresponding WT littermates. (C) Western blot analysis of $A_{2A}R$ protein levels in the striata from gb-$A_{2A}R$ KO mice (lane 1 from left), fb-$A_{2A}R$ KO mice (lane 3), and their corresponding WT littermates (lanes 2 and 4).

motor activity in MPTP-intoxicated fb-WT mice, but not in MPTP-intoxicated fb-$A_{2A}R$ KO mice (see Fig 3D). Therefore, $A_{2A}R$ antagonists act at forebrain neurons to stimulate motor activity in both normal dopamine and dopamine-depleted conditions.

Selective Deletion of the Adenosine A_{2A} Receptor in Forebrain Neurons Does Not Confer Neuroprotection against Acute MPTP-Induced Toxicity

We next examined the contribution of $A_{2A}R$s in forebrain neurons to the neuroprotection in the MPTP model. Consistent with our previous study,[12] residual striatal dopamine content of gb-$A_{2A}R$ KO mice (23.0 ± 7.4pmol/mg tissue) was significantly greater than that of gb-WT littermates (7.9 ± 1.6pmol/mg tissue) after the acute MPTP treatment (multiple injection paradigm, $p < 0.05$), confirming the neuroprotection afforded by global deletion of $A_{2A}R$s. In contrast with gb-$A_{2A}R$ KO mice, the residual striatal dopamine levels were indistinguishable between fb-$A_{2A}R$ KO mice (5.9 ± 1.8pmol/mg tissue) and fb-WT littermates (6.2 ± 1.0pmol/mg tissue) after MPTP treatment ($p > 0.05$; Fig 4). The single-injection paradigm of acute MPTP treatment produced results similar to the multiple-injection paradigm (Fig 5A). This indicates that the selectives deletion of $A_{2A}R$s in forebrain neurons is not sufficient to confer neuroprotection against MPTP-induced dopaminergic toxicity.

Intracerebroventricular Administration of the Adenosine A_{2A} Receptor Antagonist KW-6002 into fb-A_{2A} Receptor Knock-out Mice Confers Protection against Acute MPTP-Induced Neurodegeneration

We then determined whether $A_{2A}R$s located in brain cells other than forebrain neurons contribute to neuroprotection by testing the effect of ICV injection of KW-6002 on MPTP-induced neurotoxicity in fb-$A_{2A}R$ KO mice. KW-6002 (ICV) was administered into fb-$A_{2A}R$ KO mice or fb-WT littermates 10 minutes before a single injection of MPTP (40mg/kg IP). MPTP produced a significant reduction of striatal dopamine content both in fb-$A_{2A}R$ KO mice and fb-WT litter-

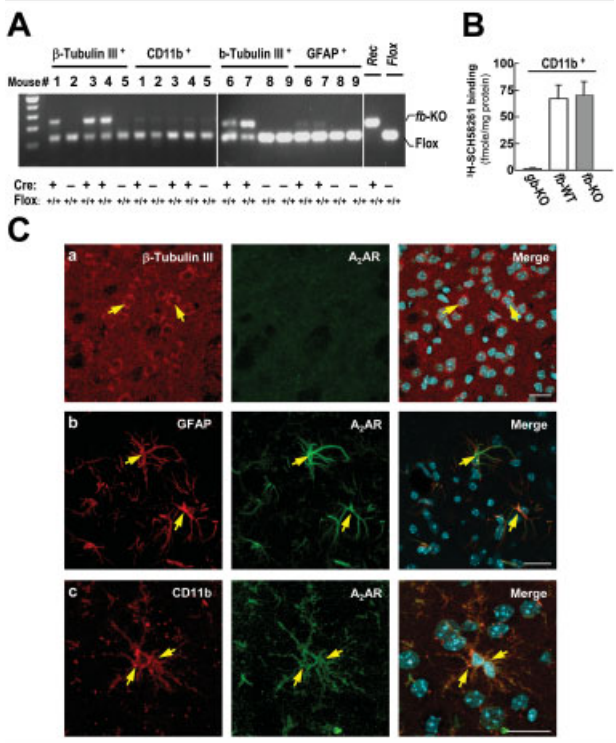

Fig 2. Selective deletion of the adenosine A_{2A} receptor ($A_{2A}R$; gene and proteins) in forebrain neurons and selective induction of $A_{2A}R$ (protein) by acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment in striatal glial cells in fb-$A_{2A}R$ knock-out (KO) mice. (A) Polymerase chain reaction (PCR) genotyping of Cre-mediated DNA recombination (where the presence of the "fb-KO" band indicates Cre-mediated $A_{2A}R$ gene deletion) in fluorescent activated cell sorted (FACS) striatal neurons (β-tubulin III$^+$), microglia (CD11b$^+$, left panel), or astrocytes (glial fibrillary acidic protein–positive [GFAP+], right panel) collected from both fb-$A_{2A}R$ KO and fb-wild-type (WT) mice. (B) Quantitative analysis of ^{3}H-SCH58261 binding densities in sorted CD11b$^+$ cells from cerebral cortex and hippocampus of gb-$A_{2A}R$ KO, fb-$A_{2A}R$ KO, and WT mice (fb-WT). gb-KO = global $A_{2A}R$ KO mice; fb-$A_{2A}R$ KO = forebrain $A_{2A}R$ KO; gb-WT = global $A_{2A}R$ wild-type mice; fb-WT = forebrain $A_{2A}R$ wild-type mice. (C) Selective colocalization of $A_{2A}R$ and glial (GFAP+ and CD11b$^+$) but not neuronal (β-tubulin III+) markers in striatum after the acute MPTP treatment, evaluated by confocal microscopy. β-Tubulin III+ (red), GFAP+ (red), and CD11b$^+$ cells (left column) were double-labeled with $A_{2A}R$ (green) (middle column) to yield merged images (yellow) (right column). Nuclei were labeled with Hoechst 33342 (light blue) and shown in the merged micrographs. Scale bars = 20μm. (A, B) Data are presented as means ± standard error (n = three to six samples/group, with CD11b$^+$ cells isolated from forebrain of three to four mice being pooled for each sample).

mates pretreated with vehicle (see Fig 5B). Pretreatment with KW-6002 protected against MPTP toxicity in fb-A_{2A}R KO mice to an extent similar to that observed in KW-6002 pretreated fb-WT littermates.

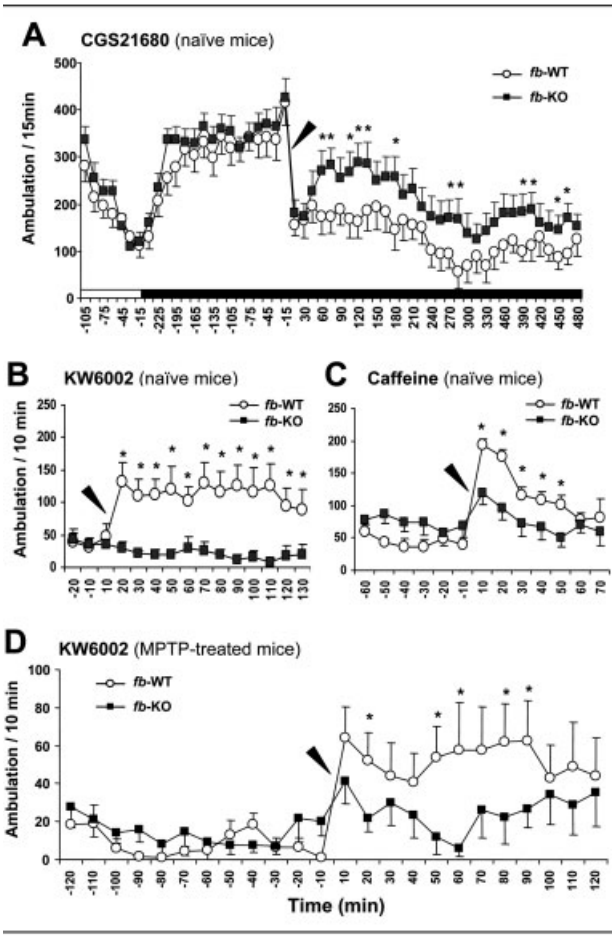

Fig 3. Selective inactivation of adenosine A_{2A} receptors (A_{2A}Rs) in forebrain neurons completely abolishes motor stimulant effects of KW-6002 and partially abolishes the motor depressant effect of CGS21680. (A) CGS21680-induced motor depression was attenuated in fb-$A_{2A}R$ knock-out (KO) mice: fb-$A_{2A}R$ KO mice (n = 15; squares) and fb-wild-type (fb-WT; circles*) littermates (n = 13) were treated with CGS21680 (0.05mg/kg intraperitoneally [IP]) and monitored for their locomotor activities using an automatic recording system during the dark phases* (closed bar) *of the light/dark cycle for 8 hours (see Materials and Methods). (B) KW-6002–induced motor stimulation in fb-WT but not in fb-$A_{2A}R$ KO mice with normal dopamine neurotransmission (n = 8–9).* Arrow *indicates the injection point. (C) Caffeine-induced motor activity in fb-WT but not fb-$A_{2A}R$ KO mice under normal dopamine neurotransmission (n = 6–8). (D) KW-6002-induced locomotor activity in fb-WT and but not in fb-$A_{2A}R$ KO mice with depleted dopamine transmission induced with subchronic treatment 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): fb-$A_{2A}R$ KO and WT mice (n = 8) were treated with MPTP (30mg/kg IP) daily for 5 days. After the fifth dose of MPTP, both fb-$A_{2A}R$ KO and fb-WT mice were recorded for basal locomotor activity for 30 minutes before they were treated with KW-6002 (3mg/kg), and recorded of their motor activities for additional 120 minutes thereafter (n = 8). *p < 0.05 comparing fb-$A_{2A}R$ KO mice with their fb-WT littermates, two-way analysis of variance followed by Bonferroni test.*

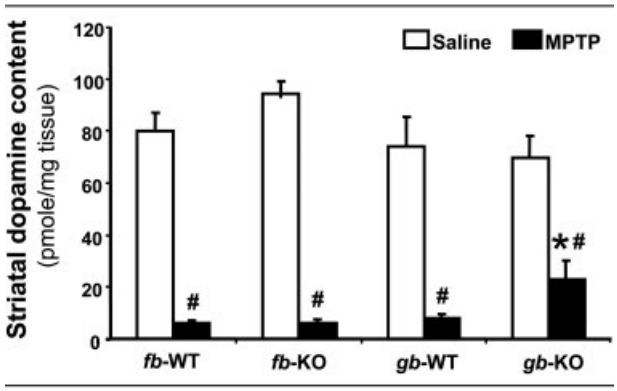

*Fig 4. Selective inactivation of adenosine A_{2A} receptors (A_{2A}Rs) in forebrain neurons does not confer neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity. Global A_{2A}R knock-out (KO) and fb-A_{2A}R KO mice and corresponding wild-type (WT) littermates (gb-WT and fb-WT) were treated with MPTP (20mg/kg three times intraperitoneally at 2-hour interval). Seven days later, striatal dopamine contents were determined by high-pressure liquid chromatography (HPLC). The striatal dopamine contents were significantly greater in gb-KO mice compared with gb-WT littermates (*p < 0.05, Mann–Whitney U *test). Striatal dopamine contents were not significantly different between fb-A_{2A}R KO mice and fb-WT littermates after MPTP treatment (*p > 0.05, Mann–Whitney U *test). n = 6–7 for the MPTP-treated group* (black bars); *n = 3 for the saline-treated group* (white bars). p < 0.05 compared to those treated with saline *p < *0.05 compared with WT littermates*

KW-6002 pretreatment in fb-A_{2A}R KO and fb-WT mice increased residual dopamine contents by twofold compared with the vehicle-pretreated groups (see Fig 5B; $p < 0.01$). Thus, KW-6002 protects against acute MPTP neurotoxicity by blocking A_{2A}Rs located in brain cells other than forebrain neurons.

MPTP treatment (single-injection paradigm) also significantly reduced the number of tyrosine hydroxylase–positive cells in substantia nigra (SN) ($p < 0.01$; see Fig 5C). This reduction was largely attenuated by pretreatment with KW-6002 (ICV) compared with the vehicle-pretreated mice ($p < 0.01$; see Fig 5C). This result suggests that A_{2A}R antagonists acting at nonforebrain cells provide not only functional protection against striatal dopamine depletion but also attenuate the degeneration of dopaminergic neurons in the SN.

Intracerebroventricular Injection of KW-6002 Attenuates Striatal Microglial and Astroglial Activation

Having established that KW-6002 affords neuroprotection by blocking A_{2A}Rs located in brain cells other than forebrain neurons, we investigated the influence of KW-6002 on glial activation in the MPTP model. Because of the elusive nature of the functional characterization of glia in the brain, we used flow cytometry analysis to quantify striatal $CD11b^+$ cells (a microglial marker) and GFAP+ cells (an astrocytic marker) in MPTP-intoxicated mice. C57BL/6 WT mice were pretreated with vehicle or KW-6002 (ICV) followed by MPTP treatment (single-injection paradigm), and the striatal cells were prepared 48 hours thereafter. MPTP treatment markedly increased the overall expression of CD11b and GFAP (per 5×10^5 total events), as the total $CD11b^+$ and GFAP+ cell numbers increased by 2- and 1.3-fold, respectively, compared with the saline-treated mice (Fig 6; $p < 0.01$). The most significant increase in $CD11b^+$ cells was seen in the subpopulation with large cell size (see Fig 6B) by more than fivefold compared with the saline-treated mice. Importantly, KW-6002 attenuated the increase in $CD11b^+$ cells induced by MPTP most significantly in the sub-

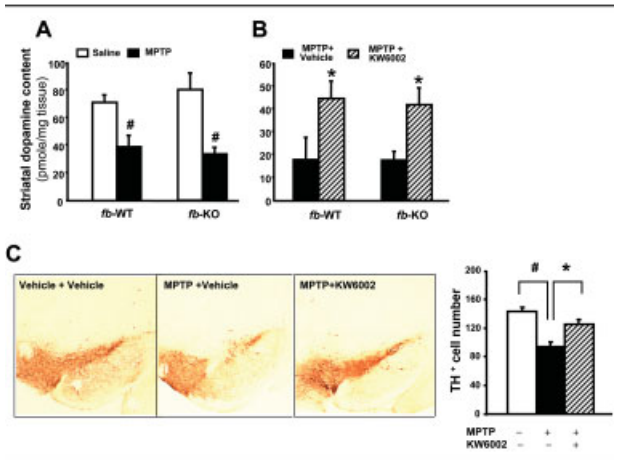

Fig 5. Intracerebroventricular (ICV) injection of KW-6002 protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity. Forebrain adenosine A_{2A} receptor (A_{2A}R) knock-out (KO) mice and fb-wild-type (WT) littermates received an ICV injection of KW-6002 (10μg/2μl/brain) 10 minutes before a single dose of MPTP (40mg/kg intraperitoneally [IP]). (A) Striatal dopamine contents in fb-A_{2A}R KO mice and fb-WT mice treated with single dose of MPTP (40mg/kg IP; black bars *or saline;* white bars) White bars *represent saline treatment. (B) Pretreatment with KW-6002 attenuated the MPTP-induced depletion of striatal dopamine contents in both fb-A_{2A}R KO and fb-WT mice compared with the vehicle-pretreated counterparts.* Black bars *represent MPTP plus vehicle;* striped bars *represent MPTP plus KW-6002.* *p < *0.05,* t *test compared with the vehicle-pretreated group, n = 6. (C) C57BL/6 WT mice were pretreated with KW-6002 (10μg/brain) or vehicle (ICV) before intoxicated with the single dose of MPTP (40mg/kg IP). The numbers of substantia nigra tyrosine hydroxylase–positive (TH+) cells (right panel) were counted 7 days after MPTP treatment.* *#p < *0.05, Bonferroni test; KW-6002–pretreated, MPTP-intoxicated group compared with vehicle-pretreated MPTP-intoxicated group, and MPTP-intoxicated group compared with group without MPTP intoxication (normal control), respectively. Representative microphotographic images (magnification ×2) from each group are showed in the left panel.*

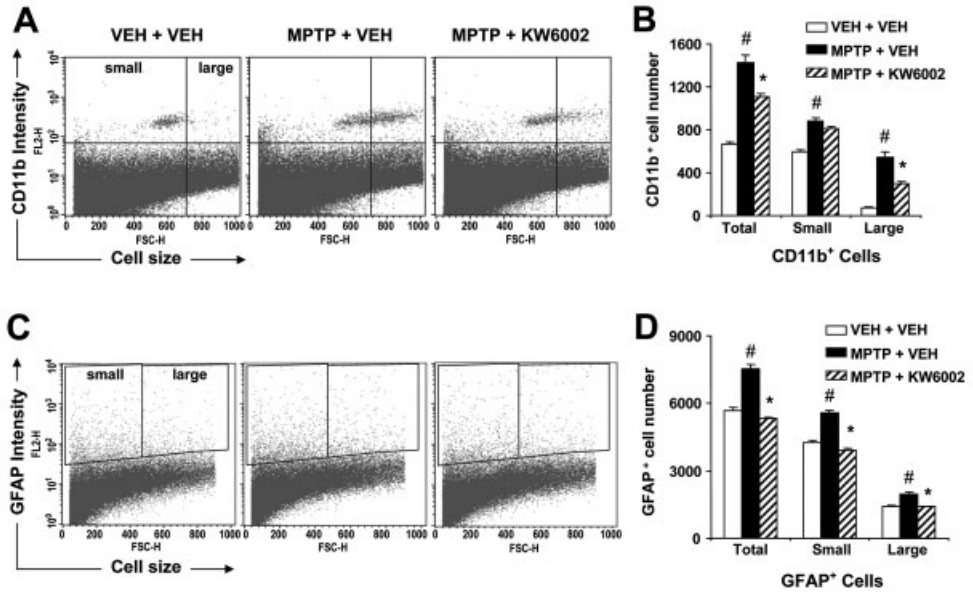

Fig 6. Intracerebroventricular (ICV) injection of KW-6002 attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced striatal glial activation assessed by flow cytometry analysis. Wild-type C57BL/6 mice were pretreated with vehicle or KW-6002 (10μg/brain ICV) 10 minutes before the single injection of MPTP (40mg/kg intraperitoneally). The striatum was dissected out at 48 hours after the MPTP treatment. The striata were acutely dissociated and labeled with anti-CD11b or anti–glial fibrillary acidic protein (GFAP) antibody, and analyzed with a fluorescent activated cell sorting (FACS) scan (see Materials and Methods). Activated microglia and astrocytes were expressed as CD11b$^+$ (A, B) or GFAP+ (C, D) per 500,000 total scanning events and further separated according to cell size. The y-axis indicates the florescence intensity, and the x-axis indicates the cell size (small vs large cells). $^{\#}p < 0.01$, Bonferroni test, compared with the saline-treated group; $^{*}p < 0.05$ compared with the vehicle-pretreated mice. $n = 4–6$/group.

population of CD11b$^+$ cells with large size (see Fig 6B; $p < 0.01$) compared with the vehicle-pretreated mice. KW-6002 pretreatment also significantly attenuated the increase of striatal GFAP+ cell number induced by MPTP treatment (see Fig 6C; $p < 0.05$) compared with vehicle-pretreated mice. This attenuation of GFAP+ cell number was observed in both large and small cell size subpopulations (see Fig 6D). These results suggest that KW-6002 can protect against MPTP neurotoxicity by attenuating MPTP-induced microglial and astroglial activation in vivo.

Discussion

The major novel finding of this study is the unambiguous demonstration that A_{2A}R antagonists stimulate motor activity and protect against dopaminergic neurotoxicity by distinct cellular mechanisms in the MPTP model of PD. The finding is strengthened by the development of fb-A_{2A}R KO mice with the forebrain regional and neuronal specificity, and near completeness of the adenosine A_{2A}R gene deletion. The A_{2A}R-mediated locomotor control was lost in mice lacking the A_{2A}R in forebrain neurons, but A_{2A}R antagonists were still able to afford neuroprotection in these mice. We also found that the neuroprotection afforded by A_{2A}R blockade against MPTP-induces toxicity correlates with the modulation of the activation of microglial and astroglial cells in the striatum.

Deletion of A_{2A}Rs in forebrain neurons abolished the motor stimulant effects of KW-6002, both in normal and dopamine-depleted conditions (see Fig 3). The A_{2A}R antagonist remained effective in producing motor stimulation in the fb-WT mice in either normal or dopamine-depleted conditions (see Fig 3). It should also be noted that while KW-6002-induced motor stimulation is completely abolished in fb-A_{2A}R KO mice, the motor-stimulant effect of caffeine and the depressant effect of CGS21680 were only partially attenuated, suggesting the possible contributions from other targets. The finding that forebrain neurons are required for the motor effects of A_{2A}R antagonists has important implications for the therapeutic development of A_{2A}R antagonists for PD patients. Patients with advanced PD (where neuroprotective strategies may not be effective because more than 90% of the dopaminergic neurons have degenerated) can still benefit from the motor stimulant effect of A_{2A}R antagonists because their molecular targets (ie, forebrain/striatal neurons) remain largely intact.

The lack of an effect on A_{2A}R-mediated neuroprotection against acute MPTP in forebrain A_{2A}R-KO mice suggests that the neuroprotection afforded by

global A_{2A}R inactivation is likely due to A_{2A}Rs located in different cell types, such as nonforebrain neurons or glial cells where A_{2A}R have also argued to be located.[23,24] KW-6002 administration (ICV) provided neuroprotection against acute MPTP-induced neurotoxicity in fb-A_{2A}R KO mice, indicating that A_{2A}Rs in brain cells other than forebrain neurons are responsible for the observed neuroprotection. This is further supported by the selective induction of the A_{2A}R in glial cells (both microglial and astrocytes) in striatum of fb-A_{2A}R KO after the acute MPTP treatment. As expected, there was no A_{2A}R immunoreactivity in the striatal neurons of fb-A_{2A}R KO mice. The A_{2A}R modulation of glial function is an important cellular mechanism by which A_{2A}R inactivation could confer neuroprotection against dopaminergic neurotoxicity, as suggested by several findings: A reactive microglial response occurs in the vicinity of dying dopaminergic neurons in human postmortem SN from PD, MPTP-intoxicated patients,[25–27] and animal models of PD[28,29]; the action of minocycline, which inhibits microglia function, attenuates degeneration of nigrostriatal dopaminergic neurons in animal models of PD[28]; and KW-6002 attenuates MPTP neurotoxicity and microglial activation in the SN.[30] Furthermore, the activation of A_{2A}Rs in astroglial cells has been implicated in excitotoxicity through its effects on the release and uptake of glutamate from astrocytes.[31,32]

Microglial activation induced by MPTP was attenuated by the A_{2A}R antagonist KW-6002 (see Fig 6), demonstrating the involvement of A_{2A}Rs in the modulation of microglial function in the MPTP model. Furthermore, flow cytometry analysis, a unique ex vivo assessment of the functional status of microglial and astroglial cells, showed that the reduction in microglial activation by KW-6002 pretreatment is largely seen in the subpopulation of microglia with large cell body size, which indicates a fully activated status (see Figs 6A, B, specifically "large" subpopulation in A). This result suggests that A_{2A}R antagonists may mainly attenuate the progression of microglial activation to fully activated status (rather than the initiation), because the large size subpopulation of MPTP-activated microglial cells are selectively attenuated by KW-6002, suggesting an important role for A_{2A}R in regulating overactivation of microglia. This was further supported by immunohistochemistry demonstration that A_{2A}R immunoreactivity was found to be colocated with activated microglia (ie, $CD11^+$ elements with the morphology of activated microglia). KW-6002 was also able to attenuate MPTP-induced astroglial activation in striatum, but in contrast with the effect on microglial activation, both large and small cell size subpopulations of astroglia were affected (see Figs 6C, D). This effect on the astroglial response may indicate that the A_{2A}R antagonist can reduce astroglial activation in different stages of activity, unlike the effect on microglia. Again, this was supported by immunohistochemistry finding that A_{2A}R immunoreactivity was found to be colocated with astrocytes (ie, GFAP+ elements). These results suggest that the glial A_{2A}Rs are important components for the A_{2A}R modulation of neurodegeneration in the MPTP model. However, the mechanisms underlying this effect await further investigation. It should be noted that such a nonneuronal-based cellular mechanism for the A_{2A}R-mediated control of neurodegeneration provides an adequate rationale to understand the broader neuroprotection afforded by A_{2A}R inactivation in diverse brain regions (with low A_{2A}R density) and against various brain injuries such as excitotoxicity, mitochondrial toxicity, and ischemic brain injury.[33,34]

In conclusion, A_{2A}R-mediated motor and neuroprotective effects are due to the presence of these receptors on different cell types. A_{2A}Rs in forebrain neurons are responsible for motor stimulation, and A_{2A}Rs in glial cells are the likely culprits involved in the neuroprotective effects observed in the acute MPTP model of PD. This identification of A_{2A}Rs in different cellular elements to control locomotion and neurodegeneration opens the real possibility of targeting these two A_{2A}R-mediated functions in PD using different dose regimens because A_{2A}R antagonists exert neuroprotection against ischemic and excitotoxic brain injuries at a dose approximately 100-fold lower than its motor stimulant doses.[35,36]

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