Cellular/Molecular

Adenosine A$_{2A}$ Receptors in Bone Marrow-Derived Cells But Not in Forebrain Neurons Are Important Contributors to 3-Nitropropionic Acid-Induced Striatal Damage as Revealed by Cell-Type-Selective Inactivation

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Endogenous adenosine acting at the adenosine A$_{2A}$ receptor (A$_{2A}$R) can modify brain injury in a variety of neurological disorder models. However, both A$_{2A}$R activation and inactivation have been shown to be neuroprotective in different situations, raising the intriguing possibility that A$_{2A}$Rs in distinct cellular elements may have different and even opposing effects. In this study, we developed three novel transgenic models to dissect out cell-type-specific actions of A$_{2A}$Rs on striatal damage by the mitochondrial toxin 3-nitropropionic acid (3-NP). Whereas global inactivation of A$_{2A}$Rs exacerbated 3-NP-induced neurological deficit behaviors and striatal damage, selective inactivation of A$_{2A}$Rs in forebrain neurons (using the Cre/loxP strategy) did not affect neurological deficit or striatal damage after the acute systemic treatment of 3-NP and intrastriatal injection of malonate. However, selective inactivation of A$_{2A}$Rs in bone marrow-derived cells (BMDCs) by transplanting bone marrow cells from global A$_{2A}$R knock-out (KO) mice into wild-type C57BL/6 mice produced a similar phenotype of global A$_{2A}$R KO mice, i.e., exacerbation of 3-NP-induced striatal damage. Thus, cell-type-selective inactivation of A$_{2A}$Rs reveals that A$_{2A}$Rs in BMDCs but not in forebrain neurons are an important contributor to striatal damage induced by mitochondrial dysfunction.

Key words: adenosine A$_{2A}$ receptor; 3-nitropropionic acid; forebrain; bone marrow-derived cells; striatum; Huntington's disease

Introduction

Adenosine levels rise markedly in response to hypoxic, traumatic, and inflammatory insults in the brain (Pedata et al., 2001). Recently, we learned that, despite the well documented neuroprotective properties of adenosine (Dunwiddie and Masino, 2001), under some circumstances, adenosine [probably acting at the A$_{2A}$ receptor (A$_{2A}$R)] may contribute to neuronal damage and death (de Mendonca et al., 2000). The potential neuroprotection by A$_{2A}$R antagonists was first reported in a global ischemia model (Gao and Phillis, 1994) and further substantiated in other cerebral ischemia and excitotoxicity models (Jones et al., 1998a,b; Monopoli et al., 1998; Popoli et al., 2002). Consistently, brain injury induced by transient focal ischemia is significantly attenuated in A$_{2A}$R knock-out (KO) mice (Chen et al., 1999). Furthermore, recent pharmacological, genetic, and epidemiological studies suggest that blockade of A$_{2A}$Rs may attenuate dopaminergic neurotoxicity (Ross et al., 2000; Ascherio et al., 2001; Chen et al., 2001) and striatal damage induced by the mitochondrial toxin 3-nitropropionic acid (3-NP) (Blum et al., 2003b; Fink et al., 2004). Thus, A$_{2A}$R inactivation offers neuroprotection against various brain insults in diverse brain regions.

However, A$_{2A}$R agonists have also been shown to offer neuroprotection under some conditions. A$_{2A}$R agonist CGS21680 [2-*p*-(2-carboxyethyl)phenethylamino-5-*N*-ethylcarboxamidoadenosine] attenuates kainate-induced hippocampal lesions and cerebral hemorrhage injury (Jones et al., 1998b; Mayne et al., 2001). Similarly, A$_{2A}$R agonist ATL-146e (4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9*H*-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester) has been shown to attenuate traumatic and ischemic injury in the spinal cord (Cassada et al., 2001). Interestingly, a recent report showed that CGS21680 attenuated neuropathological changes in R6/2 mice (a genetic model of Huntington's disease) (Chou et al., 2005). An even larger body of evidence supports the view that A$_{2A}$R activation functions as a braking mechanism to prevent excessive tissue damage after ischemic, hypoxic, and inflammatory insults in the liver, kidney, and other peripheral organs (Ohta and Sitkovsky, 2001; Day et al., 2003; Sitkovsky et al., 2004).

The fact that both A$_{2A}$R inactivation and, in some cases, A$_{2A}$R activation have been shown to provide neuroprotection implies that adenosine acting at A$_{2A}$Rs can either be detrimental or protective to neurons after brain insults. A$_{2A}$Rs are expressed at high levels in striatal neurons and at low to moderate levels in a variety of cellular elements, including glial cells, endothelium, neutrophils, and platelets (Svenningsson et al., 1999; Hettinger et al., 2001). A$_{2A}$Rs on neurons and bone marrow-derived cells (BMDCs) may produce opposite effects in brain injury. Thus, it is critical to distinguish the contribution of A$_{2A}$Rs in distinct cells in modulating striatal damage. In this study, we developed three novel transgenic mouse models to evaluate the importance of A$_{2A}$Rs in forebrain neurons versus BMDCs in modulation of 3-NP-induced striatal damage. Using an acute treatment paradigm, we found that global inactivation of A$_{2A}$Rs exacerbated 3-NP-induced striatal damage. Importantly, chimeric mice with selective inactivation of A$_{2A}$Rs in BMDCs recaptured the phenotype of global A$_{2A}$R KO mice, whereas selective inactivation of A$_{2A}$Rs in forebrain neurons did not. These results provide compelling evidence that A$_{2A}$Rs in BMDCs are critical to 3-NP-induced striatal damage, whereas A$_{2A}$Rs in forebrain neurons are not.

Materials and Methods

Generation and genotyping of global and forebrain A$_{2A}$ receptor KO mice. The generation and genotyping by PCR analysis of global A$_{2A}$R KO (gKO) mice were described previously (Chen et al., 1999; Yu et al., 2004). Congenic gKO mice on a C57BL/6 background were made by backcrossing gKO on mixed (129-Steel × C57BL/6) genetic background to C57BL/6 mice for 13–15 generations. Heterozygous cross-breeding was used to generate gKO and global wild-type (gWT) mice. The gKO and gWT mice with matched age and sex (male, 110–130 d old) were used for this study.

The generation of the forebrain-specific A$_{2A}$R conditional KO mice using the Cre/loxP strategy has been described previously (Bastia et al., 2005). Genotyping of the forebrain A$_{2A}$R KO (fKO) mice was performed by PCR analysis using a three primer set as described previously (Bastia et al., 2005). Forebrain A$_{2A}$R KO and forebrain WT (fWT) mice have been backcrossed to C57BL/6 mice for six generations to achieve a near congenic line. Because our initial analysis showed that A$_{2A}$R immunoreactivity disappeared at postnatal day 60 and A$_{2A}$R antagonist-induced motor effect had completely disappeared at postnatal day 90, respectively (E. K. Rapp and J.-F. Chen, unpublished data), the matched fKO mice and their WT littermates (male, 120–132 d old) were used in this study. In a pilot study, we noted that there were no difference among Cre(+)A$_{2A}$R$^{flox(−/−)}$, Cre(−)A$_{2A}$R$^{flox(+/+)}$, and Cre(−)A$_{2A}$R$^{flox(−/−)}$ to 3-NP induced neurotoxicity, and Cre(−)A$_{2A}$R$^{flox(+/+)}$ was used as a control for fKO mice [Cre(+)A$_{2A}$R$^{flox(+/+)}$].

Creation of chimeric mice with selective inactivation of the A$_{2A}$R in bone marrow-derived cells by transplantation. Chimeric mice were produced using bone marrow transplantation (BMT) as described previously (Yu et al., 2004). Briefly, male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) (6–9 weeks old) were irradiated with a total dose of 12.5 Grays from a ^{137}Cs source. After irradiation, the mice received transplantation of bone marrow cells (2–3 million per 0.2–0.3 ml) via tail vein injection. Bone marrow cells were isolated from female gKO mice and their WT littermates (8–10 weeks old; weight, 24–26 g) by flushing the tibia and femur with RPMI 1640 medium under sterile conditions. During each irradiation, two control mice did not receive BMT, and they died within 10 d. Seven weeks after irradiation and transplantation, we assessed the efficiency of the reconstitution of the BMDC by transplantation in chimeric mice by two independent methods. First, we took advantage of sex-mismatched BMT in the study (from female donor to male recipient or from male donor to female recipient). Seven weeks after BMT, peripheral blood mononuclear cells (PBMCs) were collected using Histopaque 1083 (Sigma, St. Louis, MO). After isolation of DNA, genotyping of sex chromosome-linked genes (*Jarid1d* and *Jarid1c*) was per-

formed as described previously (Mroz et al., 1999; Yu et al., 2004). Standard PCR conditions were used, and the annealing temperature was set at 55°C. A single primer pair (forward, 5′-TGAAGCTTTTGGCTTTGAG-3′; and reverse, 5′-CCGCTGCCAAATTCTTTGG-3′) amplifies both the X-linked (Jarid1c) and the Y-linked (Jarid1d) genes but yields different sized products, which were separated on 2% agarose gels (see Fig. 3*B*). Second, we used a substrain of C57BL/6 mice that are positive for CD45.1 (B6.SJL-*Ptprca Pep3b*/BoyJ) as the BMT donor source. The CD45.1 epitope is absent in cells of the recipient mice. Seven weeks after the transplantation, PBMCs were isolated from the chimeric mice. After washing and incubating with PE (phycoerythrin)-conjugated anti-mouse CD45.1 monoclonal antibody (BD PharMingen, San Diego, CA), subsequent flow cytometry data acquisition and analysis were performed using the flow cytometer FACSCalibur (BD PharMingen). Eight weeks after the transplantation, chimeric mice were subjected to 3-NP injection.

3-NP intoxication and assessment of neurological deficit behaviors. 3-NP (Sigma) was dissolved in saline (5 or 8 mg/ml), and the pH was adjusted to 7.4 with 1N NaOH. The solution was filtered (0.22 μm; Millipore, Bedford, MA) and kept at −80°C until use. 3-NP was intraperitoneally injected twice daily for 2 d at 12 h intervals (8:00 A.M. and 8:00 P.M.) at a dose of 60 mg/kg on the first day and 80 mg/kg on the second day (i.e., a 60–60-80–80 dose regimen).

We adopted a behavioral score system that combined the previously published methods (Gabrielson et al., 2001; Fernagut et al., 2002) to provide optimal assessment of neurological behavioral deficits after 3-NP treatment. After intoxication of 3-NP, mice were monitored for neurological deficit signs referred to as stages I, II, and III as follows. In stage I, mice were hypoactive, with reduced grooming activity and interaction with other mice but retained a normal posture and gait. In stage II, mice had marked reduction of general activity, less exploratory behavior, decreased body weight, and intermittent or intermediate clasping of hindlimbs after the mouse was grasped by the midtail. Finally, the mice in stage III were characterized by no exploratory behavior, dramatic weight loss, hindlimbs fully drawn up to the abdomen after the mouse was grasped by the midtail, or marked hindlimb dystonea with wide interlimb space, tremors, and even recumbency (moribund state).

Intrastriatal injection of malonate. Malonate was dissolved in PBS, adjusted to pH 7.4, and filtered (0.22 μm; Millipore). One microliter of PBS containing 2 μmol of malonate was injected over 10 min into the left striatum at coordinates of 0.9 mm anterior, 2 mm lateral, and 3.5 mm deep to the bregma, and the needle was left in place for 5 min before being slowly withdrawn. An equal volume of PBS was administrated to the right striatum as a control.

Histological assessment of striatal damage. Twenty-four hours after the last 3-NP injection or 7 d after malonate intrastriatal injection, mice were anesthetized with tribromoethanol (Avertin, 125 mg/kg) and perfused transcardially with saline, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were immediately removed and postfixed overnight in the same fixative and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer, pH 7.4. Sequential coronal sections (30 μm) were made on a freezing microtome, starting from the anterior aspect of the corpus callosum (bregma 1.40 mm) throughout the entire striatum (bregma −1.30 mm), according to the mouse brain atlas (Hof et al., 2000). For histological assessment, every sixth section (∼210 μm interval) was processed for cresyl violet staining or Fluoro-Jade C staining to assess cell loss and neuronal degeneration. Cresyl violet staining was performed with standard protocols. For Fluoro-Jade C staining, the method described by Schmued et al. (2005) was used (Fluoro-Jade C; Histo-Chem, Jefferson, AR)

Stereological analysis of the lesion volumes was performed by digitally acquiring cresyl violet-stained sections through the striatum at 4× objective using a computerized image analysis system (Spot Insight; Diagnostic Instruments, Sterling Heights, MI). The lesion area was characterized by extensive neuronal loss (in the case of 3-NP injection) and extensive gliosis (in the case of malonate injection), as determined on cresyl violet-stained sections. Lesion volumes were calculated by summing the cross-sectional areas of the lesion in each section and multiply-

Table 1. 3-NP-induced behavioral and pathological effects in global A$_{2A}$R KO and forebrain A$_{2A}$R KO mice and A$_{2A}$R KO→WT chimeric mice

Genotype	Neurodeficit score			Survival rate	Lesioned mice	Lesion volume (mm^3)
	Stage I	Stage II	Stage III			
Global WT	5	1	1	7/8	1/7	1.52 ± 1.52
Global A$_{2A}$R KO	1	0	5	6/8	5/6*	3.28 ± 1.38
Forebrain WT	2	1	4	7/8	4/7	2.87 ± 1.72
Forebrain A$_{2A}$R KO	4	0	4	8/8	3/8	2.33 ± 1.20
WT→WT	2	5	3	10/12	0/10	0.00 ± 0.00
A$_{2A}$R KO→WT	0	4	6	10/12	6/10**	0.79 ± 0.29***

Global A$_{2A}$R KO, forebrain A$_{2A}$R KO, and A$_{2A}$R KO→WT chimeric mice as well as their corresponding WT littermates were treated with 3-NP as described in Materials and Methods. Numerals represent the number of animals with varied neurological deficit signs at 24 h after last injection of 3-NP. Values are mean ± SE. *$p < 0.05$ compared with global WT (χ^2 test); **$p < 0.01$ compared with WT→WT (χ^2 test); ***$p < 0.01$ compared with WT→WT (Student's t test).

ing this value by the distance between sections. Fluoro-Jade C-stained sections were only assessed in the core of the striatal lesion.

Immunohistochemistry. For detecting microglial and astrocyte activation and neurotrophil infiltration in striatum after 3-NP lesioning, we used specific monoclonal antibodies against CD11b (Mac-1α chain, a marker for microglia, 1:10; BD PharMingen), glial fibrillary acidic protein (GFAP) (a marker for astrocyte, 1:100; Sigma), and Ly-6G (recognizing myeloid differentiation antigen Gr-1, a marker for neutrophil, 1:10; BD PharMingen). Brain sections (30 μm) were incubated with the antibody overnight, followed by biotin-conjugated goat anti-rat IgG-B (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and avidin–biotin complex (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). For CD11b and Ly-6G staining, peroxidase activity was localized using DAB as a chromogenic substrate (Vector Laboratories). For astrocyte staining, the sections were incubated for 72 h at 4°C and visualized under a fluorescence microscope. For IgG staining, biotinylated goat anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) was used.

Western blot. The analysis of A$_{2A}$R immunoreactivity was performed in total membranes of the striatum as described previously (Rebola et al., 2005). Briefly, the proteins isolated from the striatum were separated by SDS-PAGE (10% with a 4% concentrating gel) under reducing conditions. After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), pH 7.6, the membranes were incubated overnight at 4°C with a mouse anti-A$_{2A}$R antibody (raised against the third intracellular loop of the receptor, 1:500; Millipore, Billerica, MA) and washed with TBS-T containing 0.5% milk. The membranes were then incubated with an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:5000; GE Healthcare, Piscataway, NJ) in TBS-T containing 1% milk for 60 min at room temperature and followed up with washing in TBS-T with 0.5% milk and developed with Enhanced Chemi-Fluorescence system. The membranes were then reprobed and tested for tubulin immunoreactivity to confirm that equal amounts of protein were loaded to the gels.

Statistical analysis. Statistical comparisons between two groups were performed by using an unpaired Student's t test. The comparison of survival rate and lesioned animal rate of two genotypes was analyzed using the χ^2 test.

Results

Effects of global inactivation of the A$_{2A}$R on 3-NP-induced striatal damage

Congenic gKO mice and their WT littermates (in C57BL/6 genetic background) were tested with various treatment paradigms, including the chronic treatment regimen [i.e., 50 mg/kg, at 12 h interval, for 5 d as reported in previous studies (Fink et al., 2004)] We found that the chronic treatment paradigm did not produce clear and reproducible striatal damage. After several pilot studies, we found that the four doses of 3-NP (60−60-80−80 paradigm) resulted in clear and reproducible striatal damage with the low mortality (<25%). Consequently, we used this treatment para-

digm for the rest of our experiments. After the first two injections (60 mg/kg, i.p.), neither gKO mice nor their WT littermates showed any visible neurological deficit (behavior) symptoms. After the third injection of 3-NP (80 mg/kg), five of eight gKO mice displayed clear neurological deficit symptoms of decreased body weight, marked reduction of general activity, less exploratory behavior, and intermittent or intermediate clasping of hindlimbs after the mouse was grasped by the midtail. In contrast, seven of the eight gWT mice showed normal or slightly reduced general activity. After the fourth injection of 3-NP (80 mg/kg), most of the gKO mice (seven of eight) displayed severe (stage III) neurological deficit symptoms (i.e., no exploratory behavior, fully drawn up hindlimbs to the abdomen after being grasped by the midtail, or marked hindlimb dystonea with wide interlimb space, tremors, recumbence), whereas most of the gWT mice (five of eight) displayed only slight (stage I) neurological deficit symptoms (Table 1). However, the survival rate in gKO mice (six of eight) was similar to that of gWT mice (seven of eight) at the end of the experiment.

Consistent with behavioral results, histological evaluation showed that five of the six surviving gKO mice had bilateral striatal lesions, whereas only one of the seven surviving gWT mice had a striatal lesion ($p = 0.017$, χ^2 test). Figure 1 shows representative brain sections from gKO and gWT mice after 3-NP treatment. Cresyl violet staining demonstrates bilateral symmetrical lesioned areas in the striatum of a gKO mouse. Fluoro-Jade C staining shows neuronal degeneration. 3-NP intoxication induced striatal lesions in which degenerating soma, neuropil, and terminals were all Fluoro-Jade C positive. The Fluoro-Jade C-positive neuronal degenerative areas were consistent with the pale areas showed by cresyl violet. Similar to the behavioral results, the lesion volume in gKO mice (3.28 ± 1.38 mm^3) was apparently higher than that of gWT mice (1.52 ± 1.52 mm^3), but it did not reach statistical significance because of the unusually large lesion volume in the only damaged gWT mouse (Table 1). Nevertheless, these behavioral and histological data together suggest that global inactivation of A$_{2A}$Rs exacerbates 3-NP induced-striatal damage.

Interestingly, IgG immunoreactivity was detected in all lesioned striatum of five gKO mice (Fig. 1) and one gWT littermate after 3-NP treatment. In most of the brain sections, the areas of IgG staining were generally larger than the areas of neuronal loss shown by cresyl violet and Fluoro-Jade C staining. No IgG-positive staining was found in the unlesioned striatum of six gWT mice (Fig. 1) and one gKO mice. In some brain sections, the area of IgG staining was limited to within the lesion. Furthermore, two of the six lesioned animals exhibited histological evidence of intrastriatal hemorrhage. These results indicate an impaired blood–brain barrier (BBB) in the striatum after 3-NP treatment in both gKO and WT littermates.

Finally, we also examined the inflammatory status after 3-NP treatment by determining microglial cell activation (CD11b$^+$), astrocyte (GFAP$^+$) activation, and neutrophil infiltration (Ly-6G$^+$) using these cell-type-specific markers. Activated microglia were detected within the lateral half of the striatum, the area vulnerable to damage by 3-NP, in five of the six unlesioned gWT mice (Fig. 1*B*) and one unlesioned gKO mouse (as evidenced by

Fluoro-Jade C, cresyl violet, and IgG staining). The activated microglia showed intensified CD11b immunoreactivity, shortening and thickening of processes, and enlarged cell bodies, indicating microglial activation by 3-NP treatment. However, within the core of the lesioned area, the presence of activated microglia was significantly less, and the remaining microglia cells lost their processes (Fig. 1B). These results indicate that microglial activation proceeded (in unlesioned striatum) and was generally associated with 3-NP-induced striatal damage (in the lesioned striatum). Other than these effects associated with the striatal damage, there was no difference in microglial activation between gKO mice and their WT littermates.

Infiltrated neutropils (Ly-6G-positive cells) were only present in the core of lesioned area but not in the unlesioned striata (Fig. 1B), indicating a correlation between the degree of striatal lesion and neutrophil infiltration in these areas. GFAP-positive astrocytes were detected along the paraventricular zone and the corpus callosum but were sparse in the striatum and cortex in both lesioned and unlesioned mice (Fig. 1B).

Figure 1. Global inactivation of the A$_{2A}$R exacerbates 3-NP-induced striatal damage. A, Representative sections from gKO and gWT mice show neuronal loss as detected by cresyl violet staining, neuronal degeneration as detected by Fluoro-Jade C staining, and impaired blood brain–barrier as detected by IgG immunohistochemical staining after the 3-NP treatment. Scale bars: column 3, 0.2 mm; columns 1, 2, and 4, 1 mm. B, Representative sections from gKO mice and gWT littermates show CD11b-positive microglia, GFAP-positive astrocytes, and Ly-6G-positive neutrophil immunohistochemical staining. Arrows in B indicate the border of the lesioned area of gKO mice and the corresponding area of gWT mice. Scale bars: columns 2 and 4, 0.2 mm; columns 1 and 3, 0.5 mm.

Effects of selective inactivation of the A$_{2A}$Rs in forebrain neurons on 3-NP-induced striatal damage

In this set of experiments, we determined the contribution of A$_{2A}$Rs in forebrain neurons to 3-NP-induced striatal damage using the fKO mice in which A$_{2A}$Rs in forebrain neurons have been selectively inactivated. The generation and characterization of fKO mice has been described previously (Bastia et al., 2005). As an illustration of the selective inactivation of the A$_{2A}$R in forebrain neurons, we determined the A$_{2A}$R protein levels in the striatum and kidney by Western blot in fKO mice, gKO mice, and their corresponding WT littermates. Figure 2A shows that high levels of A$_{2A}$R protein were detected in the striata of WT littermates at the expected molecular size 42 kDa, but this band was completely abolished in fKO mice. Furthermore, disappearance of the striatal A$_{2A}$R band in fKO mice was comparable with that of gKO mice (Fig. 2A, compare lanes 3, 4 with lanes 1, 2), indicating that A$_{2A}$Rs were abolished to the background level of gKO mice. In contrast to A$_{2A}$Rs in the striatum, A$_{2A}$R protein in peripheral organs (e.g., kidney) was not affected in fKO mice. Similar levels of A$_{2A}$R proteins were detected in the kidneys of fKO mice and their WT littermates (Fig. 2A, compare lanes 7, 8 with lanes 5, 6). As expected, A$_{2A}$R proteins in the kidney were completely abolished in gKO mice.

After administration of 3-NP, both fKO mice and their WT littermates developed similar neurological deficit symptoms at various time points after the treatment. Neurological deficit scores were indistinguishable between fKO mice and their WT littermates (Table 1). Moreover, the survival rates of fKO (eight of eight) and fWT mice (seven of eight) were also comparable. Consistent with behavioral and survival results, 3-NP treatment also produced similar striatal damage in fKO mice and their WT littermates (Table 1). Three of eight fKO mice and four of seven
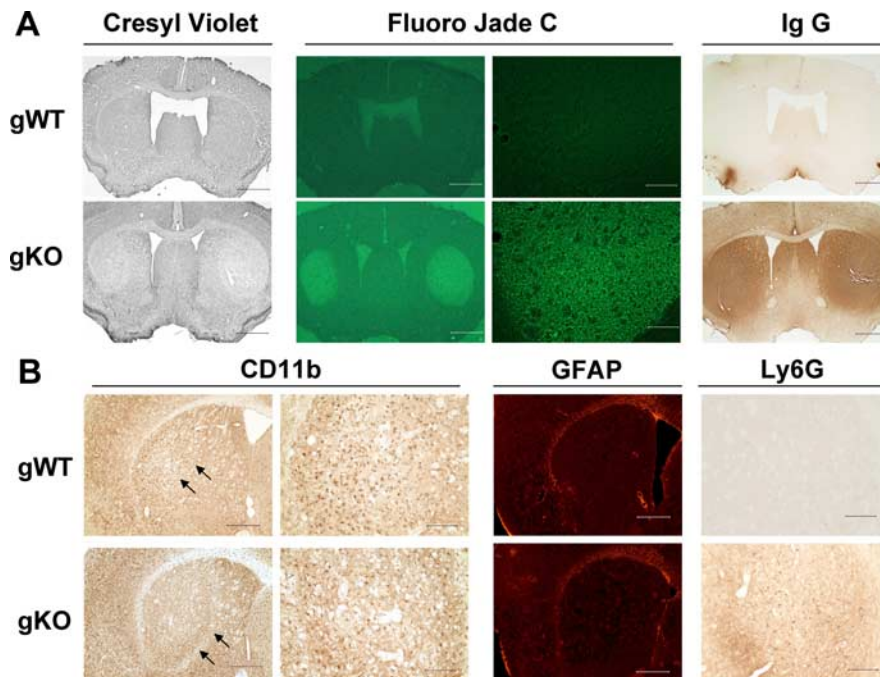
surviving fWT littermates displayed macroscopic striatal damage ($p = 0.45$, χ^2 test). Figure 2B shows representative brain sections of fKO mice and their WT littermates after 3-NP treatment. At a histological level, bilateral neuronal loss as detected by cresyl violet staining was comparable between fKO mice and their WT littermates. Furthermore, Fluoro-Jade C staining also revealed similar bilateral neuronal degeneration in fKO and their WT littermates. In addition, the extravasation of IgG was detectable in all lesioned striata and was indistinguishable between fKO mice and their WT littermates. The lesion volumes calculated from the cresyl violet-stained sections between these two groups (2.87 ± 1.72 mm^3 for fWT and 2.33 ± 1.20 mm^3 for fKO) were indistinguishable ($p = 0.79$; $n = 7–8$) (Table 1). Similar to the gKO mice, activated microglial cells were also noted in the lateral part of all three unlesioned fWT littermates and three of five unlesioned fKO mice. However, activated microglia significantly decreased within the lesioned areas in both fKO and their WT littermates (data not shown).

To further evaluate the contribution of A$_{2A}$Rs in forebrain neurons to brain injury by other mitochondrial toxins, we also determined the extent of striatal damage after intrastriatal injection of the reversible succinate dehydrogenase inhibitor malonate. Figure 2C shows representative sections of fKO and fWT mice 7 d after intrastriatal injection of malonate. GFAP staining showed a generalized increase throughout the lesioned striatum. However, the GFAP staining in the core lesion area was significantly reduced or completely abolished, indicating that malonate may also be toxic to astrocytes. Furthermore, Fluoro-Jade C-positive degenerating neurons were around the edge of lesioned area but not in the core lesioned area, indicating neuronal loss in the core lesion area after the malonate treatment. Consis-
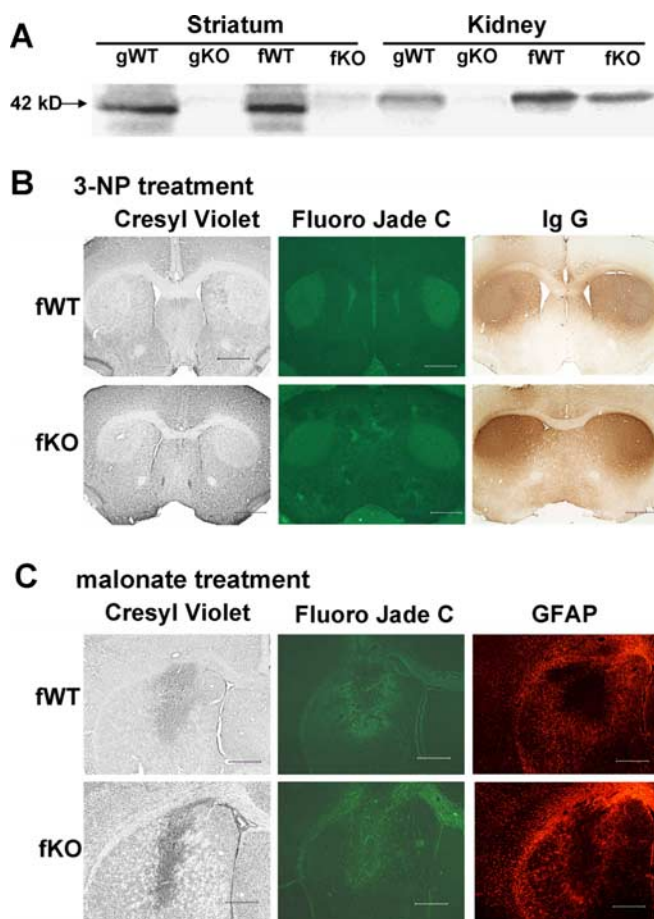
Figure 2. A$_{2A}$Rs in forebrain neurons are not critical contributors to exacerbation of 3-NP-induced striatal damage. ***A***, Western blot analysis shows that high levels of A$_{2A}$R protein were detected in striatum of fWT mice, but this band was completely abolished in fKO mice. The disappearance of striatal A$_{2A}$R band in fKO mice was comparable with the gKO mice. In contrast, A$_{2A}$R protein in kidney was not affected in fKO mice. ***B***, Representative sections from fKO and fWT mice show cresyl violet staining, Fluoro-Jade C staining, and IgG immunohistochemical staining after the 3-NP treatment. The lesioned mice and lesion volumes between these two groups were indistinguishable. Scale bars, 1 mm. ***C***, Representative photographs show cresyl violet-, Fluoro Jade C-, and GFAP-stained sections of fKO and fWT mice 7 d after the intrastriatal injection of malonate. Scale bars, 0.5 mm.

tent with the result of 3-NP-induced striatal damage, striatal damage was similar between the fKO and their WT littermates. There was no significant difference in the lesion volume induced by malonate in the fKO mice and their WT littermates (mean ± SE; 0.64 ± 0.19 mm^3 for fWT, $n = 12$; 0.79 ± 0.27 mm^3 for fKO, $n = 13$; $p = 0.45$, unpaired Student's t test).

Together, these behavioral and histological results demonstrate that selective inactivation of A$_{2A}$Rs in forebrain neurons does not affect striatal damage or neurological deficient behavior induced by 3-NP (intraperitoneal) or malonate (intrastriatal) injections.

Effects of selective inactivation of A$_{2A}$Rs in bone marrow-derived cells on 3-NP-induced striatal damage

The lack of effect of A$_{2A}$Rs in forebrain neurons on 3-NP-induced striatal damage and the demonstration of BBB impairment after 3-NP treatment prompted us to examine the contribution of A$_{2A}$Rs on peripheral inflammatory cells to 3-NP-induced striatal damage. In this set of experiments, we created chimeric mice with selective inactivation of A$_{2A}$Rs in BMDCs by transplanting BM-

DCs from gKO mice to wild-type C57BL/6 mice. The efficiency of the reconstitution of BMDCs was determined by flow cytometry analysis using CD45.1 as a donor mouse strain marker, i.e., recipient mice lacking CD45.1 received bone marrow cells from the CD45.1-positive substrain of C57BL/6 mice. Seven weeks after the transplantation, the percentage of CD45.1-positive cells was 89.1 ± 3.1% in PBMCs of chimeric mice (Fig. 3A). This result is entirely consistent with our previous report by A$_{2A}$R immunohistochemistry in PBMC of chimeric mice that ∼90% of BMDCs have been successfully reconstituted by our transplantation protocol (Yu et al., 2004). Furthermore, PCR amplification of the sex chromosome-linked genes in BMDCs from chimeric mice confirmed successful reconstitution of BMDCs. PCR amplification of the X- and Y-linked genes, *Jarid1c* and *Jarid1d*, yields different sized products, giving two bands at 300 and 330 bp for male mice and one band at 330 bp for female mice. Seven weeks after bone marrow transplantation, PCR bands of PBMCs in chimeric mice had changed from the recipient pattern to the donor pattern (Fig. 3B). Finally, Western blot analysis shows that A$_{2A}$R immunoreactivity in the striatum was not different in WT→WT mice and KO→WT mice, as expected (Fig. 3C). These results indicate that the A$_{2A}$Rs on BMDCs were successfully inactivated in KO→WT chimeric mice.

At 8 weeks after the transplantation, WT→WT mice and KO→WT mice received four doses of 3-NP. After the 3-NP treatment, both KO→WT and WT→WT mice developed similar neurological deficit symptoms (Table 1). Histological analysis revealed that whereas WT→WT mice did not display striatal lesions (0 of 10), six of ten KO→WT mice showed obvious bilateral striatal damage (vs WT→WT mice, $p < 0.01$, χ^2 test) (Table 1). Figure 3D shows representative brain sections of WT→WT and KO→WT mice after 3-NP treatment. Bilateral neuronal loss in KO→WT mice was evident by cresyl violet or Fluoro-Jade C staining. Similarly, extravasation of IgG staining was detected in the striatum corresponding to the lesioned area. In contrast, there was essentially no striatal damage in the WT→WT group, as measured by cresyl violet, Fluoro-Jade C, and IgG staining. The lesion volume calculated from cresyl violet-stained sections was significantly higher in the KO→WT group than that of the WT→WT group (Table 1). These results suggest that A$_{2A}$Rs in BMDCs are the critical contributor to the exacerbation of 3-NP-induced striatal damage in global A$_{2A}$R KO mice.

Similar to the gKO mice, activated microglial cells were also detected in the lateral portion of the unlesioned striatum in 6 of 10 from the WT→WT group (Fig. 3D) as well as three of four from the KO→WT group after the 3-NP treatment. Also, activated microglial cells were reduced in the core lesioned striatum in the KO→WT group (Fig. 3D). These results indicate that microglial activation precedes the 3-NP-induced striatal neuronal loss and that, other than their close association with striatal damage, there was no significant difference in microglial activation between the WT→WT and KO→WT groups.

Discussion

Using novel fKO mice and chimeric mice with selective inactivation of A$_{2A}$Rs in BMDCs, our study provides compelling evidence that A$_{2A}$R activity in BMDCs (not in forebrain neurons) is a critical contributor to 3-NP-induced striatal damage. We demonstrated that, whereas global inactivation of A$_{2A}$Rs exacerbates 3-NP-induced striatal damage and neurological behavioral deficits, selective inactivation of A$_{2A}$Rs in forebrain neurons does not affect either of these readouts. Importantly, selective inactivation of A$_{2A}$Rs in BMDCs by transplanting BMDCs from gKO mice to

WT mice exacerbates 3-NP-induced striatal damage. Thus, cell-type-specific genetic inactivation of A$_{2A}$Rs results in identification of A$_{2A}$Rs in BMDCs as a critical contributor to 3-NP-induced striatal damage. These results challenge a widely accepted notion that neuronal A$_{2A}$Rs in the forebrain play an important role in influencing brain injury outcomes and reinforce the important role of A$_{2A}$Rs in BMDCs in modulating brain injury. Dissection of distinct actions by A$_{2A}$Rs in BMDCs and forebrain neurons will facilitate the development of A$_{2A}$R-based therapeutic strategies for neurological disorders such as Huntington's and Parkinson's diseases.

Different 3-NP intoxication outcomes in global A$_{2A}$R KO mice may reflect complex actions of A$_{2A}$Rs in different cellular elements

Several groups, including ours, have provided results supporting the notion that A$_{2A}$R antagonists reduce striatal damage induced by excitotoxicity and mitochondrial toxins (for review, see Cunha, 2005; Fredholm et al., 2005). However, several recent studies strongly suggest that A$_{2A}$R modulation of brain injury is complex, depending on the nature of insults and the treatment paradigm (Blum et al., 2003b; Tebano et al., 2004). 3-NP-induced striatal damage can be exacerbated or attenuated in gKO mice, depending on the dose administered and the administration schedule (Blum et al., 2003b). Similarly, we observed that 3-NP-induced striatal damage was significantly enhanced in gKO mice (Fig. 1, Table 1), in contrast to our previous report of the attenuation in gKO mice (Fink et al., 2004). It should be noted that we used different 3-NP treatments (60–60–80–80 mg/kg at 12 h interval) compared with the regimen in the previous study (daily treatment with 50 mg/kg 3-NP at 12 h interval for 5 d). The change in treatment paradigm was attributable to the use of congenic A$_{2A}$R KO mice in C57BL/6 background, which differ from the gKO mice in a mixed C57BL/6 × 129-Steel or pure 129-Steel background used in the previous study (Fink et al., 2004). However, several pilot studies using congenic A$_{2A}$R KO mice with the same 3-NP paradigm used in the previous study (Fink et al., 2004) failed to produce significant striatal damage. Taking Blum's findings into consideration, we tested various 3-NP treatment paradigms and found that injection of 3-NP (the 60–60–80–80 mg/kg dose regimen at 12 h interval) resulted in clear striatal damage with <25% mortality. Using this protocol, we found that gKO mice were more susceptible to 3-NP than their WT littermates. The different effects of global A$_{2A}$R inactivation in 3-NP-induced striatal damage with various treatment paradigms (the present and previous studies) may reflect complex or sometimes opposing effects of A$_{2A}$Rs in different cellular elements. For example, modulation of glutamate release by neuronal A$_{2A}$Rs and of neuroinflammation by A$_{2A}$Rs of the BMDCs may produce opposite outcomes of A$_{2A}$R inactivation on striatal damage. Thus, it is critical to dissect out the cell-type-specific actions of A$_{2A}$Rs in the 3-NP intoxicated
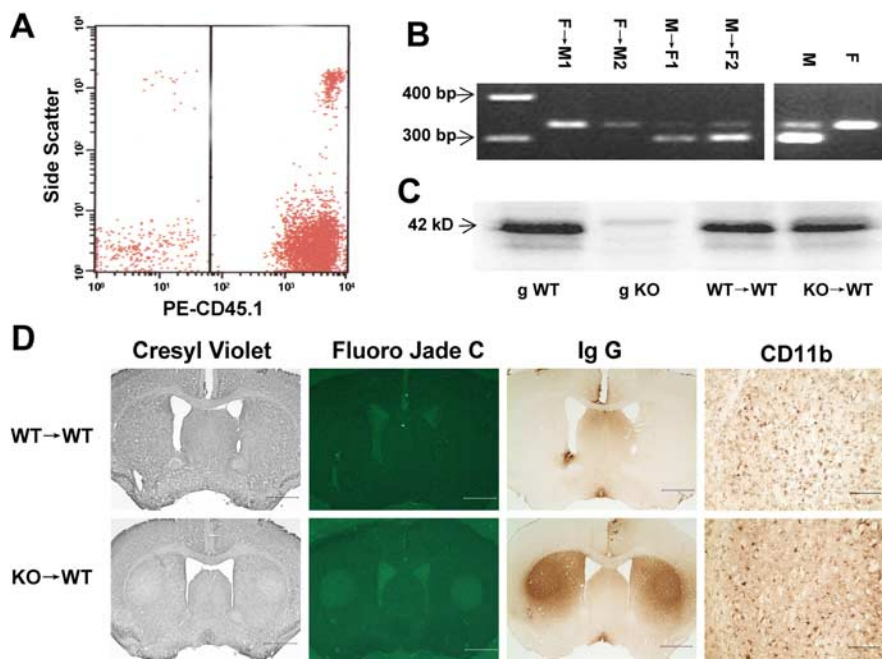

Figure 3. A$_{2A}$Rs in bone marrow-derived cells contribute critically to exacerbation of 3-NP-induced striatal damage. **A**, Reconstitution of BMDCs in recipient mice after BMT. Irradiated mice in CD45 gene background received BMDCs from CD45.1-positive animals. Seven weeks after transplantation, PBMCs were isolated and examined by fluorescence-activated cell sorting for CD45.1-positive cells. The percentage of CD45.1-positive cells in PBMCs is 89.1 ± 3.1% (n = 8), indicating efficient reconstitution of BMDCs in chimeric mice. **B**, PCR amplification of the X- and Y-linked genes *Jarid1c* and *Jarid1d* yields two bands at 300 and 330 bp for male (M) and one band at 330 bp for female (F). Seven weeks after BMT, PCR bands of PBMCs changed from the recipient pattern to the donor pattern: lanes 1, 2, two male recipients (female donor, F→M); lanes 3, 4, two female recipients (male donor, M→F). **C**, Western blot analysis shows that A$_{2A}$R immunoreactivity in striatum was not different in WT→WT mice and KO→WT mice. **D**, Representative sections of cresyl violet and Fluoro-Jade C staining and IgG and CD11b immunohistochemistry in WT→WT and KO→WT mice after the 3-NP treatment. Scale bars: columns 1–3, 1 mm; column 4, 0.2 mm.

striatum before an adenosine-based therapeutic strategy can be considered.

A$_{2A}$Rs in forebrain neurons are not a critical contributor to the exacerbation of 3-NP-induced striatal damage

It has been widely accepted that A$_{2A}$Rs may influence the outcome of brain injury by modulating glutamate and aspartate release in various brain regions under different pathological conditions (Dunwiddie and Masino, 2001; Latini and Pedata, 2001). A$_{2A}$R agonists enhance the glutamate release under normal and pathological conditions (O'Regan et al., 1992; Latini and Pedata, 2001; Marcoli et al., 2003). This facilitating effect by A$_{2A}$R agonists has been primarily attributed to an effect on glutamatergic terminals (Nikbakht and Stone, 2001; Rosin et al., 2003). To our surprise, selective inactivation of A$_{2A}$Rs in forebrain neurons failed to affect striatal damage after the acute systemic 3-NP treatment paradigm. Moreover, direct injection of malonate into the striatum, thus avoiding potential systemic effects from intraperitoneal injection, produced similar striatal damage in fKO mice and their WT littermates. This result obtained using the acute 3-NP treatment paradigm argues that selective deletion of A$_{2A}$Rs in forebrain neurons (presumably also abolishing A$_{2A}$R-mediated facilitation of glutamate release) does not affect the sensitivity of striatal neurons to mitochondrial toxins. The result is consistent with our recent finding that selective inactivation of A$_{2A}$Rs in the forebrain abolished CGS21680-mediated facilitation of glutamate release in synaptosomes but still failed to confer neuroprotection against ischemic brain injury (L. Yu and Chen, unpublished data). Thus, A$_{2A}$R-mediated facilitation of gluta-

mate release at presynaptic sites does not seem to play a critical role in 3-NP-induced brain injury.

This interpretation is, however, limited by the potentially opposing effects of A$_{2A}$Rs in presynaptic and postsynaptic neurons. Recent studies showed that blockade of postsynaptic A$_{2A}$Rs is detrimental to striatal neurons as demonstrated by amplified NMDA current, increased intracellular calcium efflux, and enhanced hippocampal excitoxicity after exposure to excitotoxins (Robledo et al., 1999; Popoli et al., 2002; Tebano et al., 2004). Thus, A$_{2A}$R inactivation may not only exert beneficial effects by reducing glutamate levels at presynaptic sites but may also produce potentially detrimental effects by modulating NMDA receptor-mediated currents at postsynaptic sites (Blum et al., 2003a). Because fKO mice inactivate both presynaptic (corticostriatal neurons) and postsynaptic (intrinsic striatal neurons) striatal neurons, the lack of the effect of A$_{2A}$Rs in forebrain neurons may result from a delicate balance between presynaptic and postsynaptic actions. Additional experiments are needed to clarify the specific effect of A$_{2A}$Rs at presynaptic and postsynaptic sites.

A$_{2A}$Rs in bone marrow-derived cells contribute critically to the exacerbation of 3-NP-induced striatal damage

The failure of forebrain-specific inactivation of A$_{2A}$Rs to produce the exacerbation of 3-NP-induced striatal damage present in gKO mice suggests that A$_{2A}$Rs in cells other than forebrain neurons may play a critical role in modulating 3-NP-induced striatal damage. A study by Jones et al. (1998b) suggests that A$_{2A}$R agonists might work at peripheral A$_{2A}$Rs because only peripheral injection (but not local intrahippocampal injection) of CGS21680 reduces kainate-induced hippocampal lesions. The possibility of a contribution by peripheral A$_{2A}$Rs has been strengthened by our finding that IgG staining correlates well with the striatal damage area in the 3-NP-treated striatum, indicating BBB impairment after 3-NP treatment. Together with our recent finding of the critical contribution of A$_{2A}$Rs in BMDCs to modulation of transient ischemic injury (Yu et al., 2004), these lines of evidence prompted us to evaluate the contribution of the A$_{2A}$R in BMDCs to 3-NP-induced striatal damage using chimeric mice with selective inactivation of A$_{2A}$Rs in BMDCs. Using this novel model, we found that, after challenge with 3-NP, none of the WT→WT mice displayed any striatal lesions, whereas 60% of the surviving KO→WT mice showed obvious bilateral striatal damage. Although similar exacerbation was not noted at the neurological behavioral level in KO→WT mice, this discrepancy may be attributable to the overall smaller striatal damage in the KO→WT groups, which is likely attributable to an immunosuppressive effect, as suggested in a previous study showing protection against spinal cord injury by BMT (Kipnis et al., 2004). Nonetheless, this result suggests that A$_{2A}$Rs on BMDCs are critical to the modulation of 3-NP-induced striatal damage observed in gKO mice. The mechanism by which selective inactivation of A$_{2A}$Rs in BMDCs exacerbates 3-NP-induced striatal damage is not clear. However, A$_{2A}$R-mediated vascular effects, including vasodilation (Phillis, 2004), inhibition of platelet aggregation (Sandoli et al., 1994), and suppression of super-oxygen species generation by neutrophils (Cronstein et al., 1990), may provide partial explanations for the exacerbation of 3-NP-induced striatal damage in the KO→WT mice. The finding of BBB dysfunction in 3-NP-intoxicated mice raises the possibility that A$_{2A}$Rs on BMDCs may affect 3-NP-induced striatal damage through their influence on the BBB. Additional experiments are required to

clarify the exact role of the A$_{2A}$R in BMDCs in this pathological process.

Although our studies using three different A$_{2A}$R KO genotypes reveal an important role for A$_{2A}$Rs in BMDCs in modulation of 3-NP-induced striatal damage, A$_{2A}$Rs in glial cells may also contribute critically to the modulation of 3-NP-induced striatal damage. A contribution of A$_{2A}$Rs in glial cells would be consistent with the finding that selective inactivation of A$_{2A}$Rs on forebrain neurons did not affect 3-NP-induced striatal damage, whereas the global inactivation of A$_{2A}$Rs exacerbated the 3-NP-induced striatal damage. Our immunohistochemical analysis of glial activation and neutrophil infiltration indicate that microglial activation precedes striatal damage (as detected by Fluoro-Jade C staining and cresyl violet staining) but was significantly reduced in the core lesioned area after striatal damage. Other than these general observations, it is not clear how A$_{2A}$Rs influence microglial cells and neutrophils and consequently the striatal damage. Additional experiments involving different time points with glial cell-specific A$_{2A}$R manipulation are required to clarify the exact role of the glial A$_{2A}$R in the modulation of 3-NP-induced striatal damage.

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