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**Cellular Mechanisms for Motor Stimulation and Neuroprotection by A2A Antagonists in PD model**

1. **Specific Aims** – not significantly modified.

2. **Main findings and Significances:**

Specific Aim 1: 
Hypothesis: A\(_{2A}\)R antagonists modulate motor activity and neuroprotection by acting at forebrain neurons and non-forebrain neurons, respectively.

Specific Aim 2: 
Hypothesis: A\(_{2A}\)Rs act at striatal, cerebral cortical and substantial nigral neurons to modulate MPTP-induced dopaminergic neurotoxicity.

Specific Aim 3: 
Hypothesis: A\(_{2A}\)R antagonists act at peripheral and brain inflammatory cells to exert neuroprotection against MPTP neurotoxicity.

C2. **Forebrain- and neuron-specific deletion of the A\(_{2A}\)R gene in forebrain-A\(_{2A}\)R KO mice.**

To generate forebrain-specific A\(_{2A}\)R KO (fb-KO) mice, we cross-bred floxed A\(_{2A}\)R mice to Cre-CaMKII\(\alpha\) transgenic mice in which Cre proteins are expressed under the control of an 8.5 kb promoter fragment of the CaMKII\(\alpha\) gene. Using PCR primers targeted to the floxed A\(_{2A}\)R genes, we detected CaMKII\(\alpha\)-Cre mediated A\(_{2A}\)R gene deletion ("KO" band) in Cre(+) mice, specifically in forebrain regions including striatum (ST), cerebral cortex (CT), hippocampus (HIP) and olfactory bulb (OB), but not in cerebellum (CB), brainstem (BS), or midbrain (MB) where dopaminergic neurons reside, or in six peripheral organs tested (Figure C2-A). A residual floxed band was still present in the forebrain, likely due to the A\(_{2A}\)R in glial cells that do not express CaMKII\(\alpha\)-Cre.

Both \(^{3}\text{H}\)-SCH58261-binding (Figure C2-B) and western blot analyses (Figure C2-C) demonstrated the abolishment of A\(_{2A}\)R proteins in fb-KO to the background level observed in global-A\(_{2A}\)R KO mice (gb-KO). To further characterize the cellular specificity of the A\(_{2A}\)R gene deletion, we separated neurons (\(\beta\)-tubulin III\(^+\) cells), microglia (CD11b\(^+\) cells), and astrocytes (GFAP\(^+\) cells) from three fb-KO and two fb-WT mice (all of them with the floxed A\(_{2A}\)R) by FACS. The "KO" band was detected in the neuronal preparations from all three fb-KO mice but was not observed in the microglial or astroglial preparations from the same fb-KO mice (Figure C1-D). A residual floxed band was still present in the neuronal preparations, which may suggest the presence of striatal neurons that do not express CaMKII\(\alpha\)-Cre (e.g. striatal cholinergic interneurons). Additionally, \(^{3}\text{H}\)-SCH58261- binding confirmed that A\(_{2A}\)R protein on microglial cells is not affected in these mice (Figure C2-E). Together, these data clearly demonstrate at the gene and protein levels that A\(_{2A}\)Rs are completely depleted in forebrain neurons in the fb-KO line.
C4. Genetic deletion of A2ARs in forebrain neurons abolishes the psychomotor effect of A2AR antagonists, but does not confer neuroprotection against MPTP neurotoxicity

We evaluated the effect of the genetic deletion of A2ARs in forebrain neurons on the A2AR modulation of psychomotor activity. We assessed the motor stimulant effect of the selective A2AR antagonist KW-6002 and of the non-selective antagonist caffeine in fb-A2AR KO mice. In accordance with previous studies, KW-6002 (3 mg/kg) produced a strong motor stimulation in WT mice, which was completely abolished in fb-A2AR-KO mice (Figure C4A). Similarly, caffeine (10 mg/kg) produced a strong motor stimulation in WT mice, which was largely attenuated in fb-A2AR KO mice (Figure C4B), as occurs in gb-A2AR KO mice. Thus, A2ARs in forebrain neurons are critical for the A2AR-mediated motor effects. We also examined the effect of global or forebrain A2AR inactivation on MPTP-induced dopaminergic toxicity. The residual striatal dopamine levels were indistinguishable between fb- A2AR KO mice and their WT littermates after a single injection of MPTP (40 mg/kg x1, i.p.) (Figure C4C) or three injections of MPTP (20 mg/kg x 2 at 2 hr interval, i.p) (Figure 4D). In contrast, residual dopamine content in the striatum of gb-A2AR KO mice was significantly higher than that of WT littermates after MPTP treatment (Figure C4D, *P<0.05), consistent with our previous study. Thus, deletion of A2ARs in forebrain neurons does not confer neuroprotection against MPTP-induced brain dopaminergic toxicity.

C7. Intraventricular administration of the A2AR antagonist KW-6002 into forebrain A2AR-KO mice protects against MPTP neurotoxicity.

To determine if A2ARs located in brain cells other than forebrain neurons contribute to neuroprotection, we tested the effect of intracerebroventricular (icv) injection of KW-6002 on MPTP-induced dopaminergic toxicity in fb-KO mice. We reasoned that if A2ARs in forebrain neurons were the only targets of KW-6002, then KW-6002 would not be able to provide neuroprotection in fb-KO mice. If instead, KW-6002 afforded neuroprotection, it would act on A2ARs located in brain cells other than forebrain neurons. Vehicle or KW-6002 (10 µg/2 µl/brain) was administered into the left ventricle of fb-KO mice or WT littermates 10 min prior to a single injection of MPTP (40 mg/kg, i.p.). We measured dopamine content 7 days after MPTP intoxication and found that MPTP produced about a 50% reduction of striatal dopamine content in fb-KO mice and WT littermates pre-treated with vehicle (Figure C7-A). Pre-treatment with KW-6002 protected against MPTP-induced toxicity in fb-KO mice to an extent similar to that observed in WT littermates (Figure C7-A). KW-6002 also attenuated MPTP-induced reduction of TH+ cells in the substantia nigra in fb-KO and WT mice (Figures C7-B & C7-C). Thus, protection against MPTP-induced neurotoxicity afforded by KW-6002 is likely due to the blockade of A2ARs in brain cells other than forebrain neurons.
C8. KW-6002 markedly reduced MPTP-induced microglial activation in striatum.

To seek further evidence for the role of A2ARs in microglial cells in PD models, we investigated the influence of KW-6002 on microglial activation in the MPTP model. To better characterize microglial function in vivo, we have developed flow cytometry analysis to quantify striatal CD11b+ cells in different proliferation and activation stages in MPTP-intoxicated mice. C57BL/6 WT mice were pretreated with vehicle or KW-6002 (icv) followed by MPTP treatment (40 mg/kg, ip). The striatum was dissected, dissociated, and labeled with fluorescent CD11b antibody 48 hrs after MPTP treatment. MPTP treatment increased the total CD11b+ cell numbers by 2-fold (per 5 x 10⁵ total events) compared to saline treatment (Figures C8-A & C8-B). KW-6002 attenuated the MPTP-induced increase in CD11b+ cells. Interestingly, the most significant MPTP-induced increase in CD11b+ cells was seen in the subpopulation with a large cell size (Figure C8-B). In addition, it is in this subpopulation of large diameter CD11b+ cells that the MPTP effect is significantly attenuated by KW-6002. Thus, A2AR antagonists may attenuate the over-activation rather than the initial proliferation of microglial cells. These results suggest that KW-6002 may confer neuroprotection against MPTP by attenuating microglial responses in vivo.


To investigate the identity of the brain cells other than forebrain neurons by which KW-6002 affords neuroprotection, we examined A2AR immunoreactivity (IR) in microglial cells by double immunolabeling in MPTP-intoxicated fb-KO mice 48 hours after MPTP treatment (Figure C9). CD11b+ activated microglial cells demonstrating the characteristic cellular morphology were detected in the striatum (Figure C9-lower left panel). Double-labeling revealed that MPTP markedly induced A2AR-IR in microglia (Figure C9-lower panels). As expected, no A2AR-IR was detected in β-tubulin-III+ neurons in the striatum of fb-KO mice after acute MPTP treatment (Figure C9-upper middle panel). Thus, MPTP treatment markedly induces A2AR expression in microglial cells in the striatum.

3. Plans:

Specific Aim 2:

(1) Despite of the lack of neuroprotection in forebrain A2AR KO mice, we are evaluating the effect of selective inactivation of the A2AR in striatal neurons on MPTP-induced MPTP neurotoxicity since striatal A2ARs and cortical A2ARs have been to exert opposite effects on excitotoxicity and psychomotor behavior.

(2) We are also performing intra-nigral injection of A2AR antagonists to localize the anatomical locus whereby A2AR antagonists exert neuroprotective effects.
Specific Aim 3:

(1) We are performing bone marrow transplantation experiment to create chimeric mice with selective inactivation and reconstitution of the A2AR activity in bone marrow derived cells. Using these chimeric mice, we will critically evaluate the effect of selective manipulation of A2ARs in bone marrow-derived cells on MPTP-induced dopaminergic neurotoxicity.

(2) We are developing microglia-specific A2A receptor knockout mice to critically evaluate the role of A2ARs in microglial cells on MPTP-induced dopaminergic neurotoxicity.

4. Publications: