Published in final edited form as:

Biol Psychiatry. 2014 June 1; 75(11): 855–863. doi:10.1016/j.biopsych.2013.05.003.

Regulation of fear responses by striatal and extra-striatal adenosine A_{2A} receptors in forebrain

Catherine J. Wei¹, Elisabete Augusto^{1,2,5}, Catarina A. Gomes², Philipp Singer^{3,4}, Yumei Wang¹, Detlev Boison³, Rodrigo A. Cunha^{2,5}, Benjamin K. Yee^{3,4}, and Jiang-Fan Chen¹

¹Molecular Neuropharmacology Laboratory, Department of Neurology, and Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts 02118 ²CNC- Center for Neurosciences of Coimbra, University of Coimbra, 3004-517 Coimbra, Portugal ³R.S. Dow Neurobiology Laboratories, Legacy Research Institute, Portland, Oregon 97232 ⁴Laboratory of Behavioural Neurobiology, Swiss Federal Institute of Technology Zurich, Schorenstrasse 16, CH 8603 Schwerzenbach, Switzerland ⁵Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal

Abstract

Background—Adenosine A_{2A} receptors (A_{2A}Rs) are enriched in the striatum but are also present at lower levels in the extra-striatal forebrain (*i.e.*, hippocampus, cortex), integrating dopamine, glutamate, and brain-derived neurotrophic factor (BDNF) signaling, and are thus essential for striatal neuroplasticity and fear and anxiety behavior.

Methods—We tested two brain region-specific A_{2A}R knockout lines with A_{2A}Rs selectively inactivated either in the striatum only (st-A_{2A}R KO) or the entire forebrain (striatum, hippocampus, and cortex, fb-A_{2A}R KO) on fear and anxiety-related responses. We also examined the effect of hippocampus-specific A_{2A}R deletion by local injection of AAV5-Cre into conditional (floxed)-A_{2A}R knockout mice.

Results—Selective deletion of striatal A_{2A}Rs in st-A_{2A}R KO mice increased Pavlovian fear conditioning (both context and tone), but when the A_{2A}R deletion was extended to include extra-striatal regions in fb-A_{2A}R KO mice, context fear conditioning was normalized and tone fear conditioning was attenuated. Moreover, focal deletion of hippocampal A_{2A}Rs by AAV5-Cre injection selectively attenuated context (but not tone) fear conditioning. Deletion of A_{2A}Rs in the entire forebrain in fb-A_{2A}R KO mice also produced an anxiolytic phenotype in both the elevated plus maze and open field tests and increased the startle response. These extra-striatal forebrain A_{2A}R behavioral effects were associated with reduced BDNF levels in the fb-A_{2A}R KO hippocampus.

Correspondence to: Jiang-Fan Chen at Department of Neurology, Boston University School of Medicine, 715 Albany Street, C329, Boston, MA 02118, USA. Phone: 617-414-1249, Fax: 617-638-5354, chenjf@bu.edu.

DISCLOSURES/CONFLICT OF INTEREST

The authors declare no conflict of interest.

Conclusions—This study provides the first evidence that inactivation of striatal A_{2A}Rs facilitates Pavlovian fear conditioning while inactivation of extra-striatal A_{2A}Rs in the forebrain inhibits fear conditioning and also affects anxiety-related behavior.

Keywords

Adenosine A_{2A} receptor; Fear conditioning; Anxiety; Startle Response; Striatum; Hippocampus; Cortex; BDNF

INTRODUCTION

Conditioned fear predicts aversive events from prior experience and its dysfunction leads to maladaptive fear responses that underlie neuropsychiatric disorders such as post-traumatic stress disorder (PTSD). Adenosine A_{2A} receptors (A_{2A}Rs) are abundantly expressed in striatopallidal neurons where they interact with dopamine, glutamate and CB1 receptor signaling to modulate striatal neuroplasticity underlying mnemonic and mood-related behaviors (1). A_{2A}Rs are also present in the extra-striatal forebrain, namely in the hippocampus and cortex (2), where they partake in hippocampal (3, 4) and corticostriatal synaptic plasticity (5, 6). The ability of A_{2A}Rs to control brain-derived neurotrophic factor (BDNF) levels and to transactivate their TrkB receptors emerges as a candidate mechanism for A_{2A}R-dependent modulation of emotional behavior (7). Thus, A_{2A}Rs are uniquely positioned to modulate multiple parallel pathways implicated in mnemonic and emotional processes (8, 9). However, it is not yet established if A_{2A}Rs can control fear behavior, and the limited studies on the impact of pharmacological (10–13) or genetic manipulations of A_{2A}Rs (13, 14) on avoidance responses or anxiety have produced largely inconclusive results. This is attributed to the difficulty in disentangling the: (i) different contributions of distinct brain regions to emotional responses; (ii) potentially different effects of A_{2A}Rs on different emotional processes such as conditioned fear and anxiety; and (iii) different effects of A_{2A}Rs in distinct brain regions on brain plasticity related to cognition and emotion. The latter is particularly important since A_{2A}Rs in distinct forebrain regions (such as striatum *versus* hippocampus and cerebral cortex) exert an opposite control over psychomotor responses (15).

To molecularly dissect the striatal and extra-striatal contributions of forebrain A_{2A}Rs to emotional processes, we analyzed conditioned and innate fear responses in two conditional A_{2A}R knockout (KO) lines: st-A_{2A}R KO mice with deletion of A_{2A}Rs restricted to the striatum and fb-A_{2A}R KO mice with A_{2A}R deletion extended beyond the striatum to the entire forebrain, including cerebral cortex and hippocampus. We found that A_{2A}R deletion in striatal neurons enhanced context and tone fear conditioning without affecting anxiety-like behavior. Deleting the A_{2A}R in the entire forebrain or focal deletion of hippocampal A_{2A}R normalized or attenuated context and tone fear conditioning while also producing an anxiolytic phenotype. We conclude that striatal and extra-striatal A_{2A}Rs may exert opposite control over fear conditioning, prompting consideration of forebrain A_{2A}Rs as novel therapeutic targets to manage maladaptive fear responses.

METHODS AND MATERIALS

All procedures were approved by the IACUCs at Boston University School of Medicine, the Legacy Research Institute, the Cantonal Veterinary Office in Zurich, and the Faculty of Medicine of the University of Coimbra.

Generation of forebrain-specific and striatum-specific A_{2A}R knockout mice

Forebrain-specific A_{2A}R knockout mice [fb-A_{2A}R KO, CaMKII-Cre(+)A_{2A}R^{flox/flox}, near *congenic* >97% C57BL/6J genetic background, for both fb-A_{2A}R KO and fb-WT (CaMKII-Cre(-)A_{2A}R^{flox/flox})] were generated as previously detailed (15–17). Striatum-specific A_{2A}R knockout mice [st-A_{2A}R KO, Dlx5/6-Cre(+)A_{2A}R^{flox/flox}, F5 generation of a *mixed* 129-Steel-C57BL/6-FVB genetic background, for both st-A_{2A}R KO and st-WT (Dlx5/6-Cre(-)A_{2A}R^{flox/flox})] mice were also generated as previously described (15, 17). Fb-A_{2A}R KO and st-A_{2A}R KO mice were characterized by their selective deletion of A_{2A}Rs in the forebrain or exclusively in striatal neurons, as validated by: (i) X-gal staining of a Cre-expressing Rosa26 reporter transgenic line; (ii) PCR analysis of Cre-mediated A_{2A}R deletion; (iii) A_{2A}R immunohistochemistry localization; and (iv) A_{2A}R binding with ³H-ZM241385 (15, 16, 18, 19).

Intra-hippocampal injection of AAV5-Cre into conditional (floxed)-A_{2A}R mice

Either AAV5-CMV-Cre-GFP (Vector BioLabs, 2 µl of 1×10¹² genome copies/mL) or its control viral vector (AAV5-CMV-GFP) were injected stereotactically into both hippocampi of conditional (floxed)-A_{2A}R adult mice (AP=−2.5 mm from bregma, ML=±2.0 mm from midline, and DV=−1.5 mm from the skull surface). Mice were tested for conditioned freezing three weeks later.

Fluorescence immunohistochemistry

Mice were anesthetized with Avertin [2% 2,2,2-tribromoethanol and 1% amyl alcohol]. Brains were transcardially perfused with 4% paraformaldehyde in PBS, post-fixed, and cryopreserved. 30-µm coronal sections (Leica Microsystems) were incubated for 1 h in PBS containing 0.25% Triton X-100 and 5% donkey serum, followed by incubation with mouse anti-mouse NeuN (1:1,000; Millipore) and rabbit anti-GFP (1:1,000; Abcam) antibodies overnight at 4°C. Sections were rinsed 3×10 min in PBS and then incubated with donkey anti-mouse and donkey anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 or 555 (1:200; Invitrogen) for 1 h at room temperature. Sections were rinsed, mounted with Vectashield mounting medium, and examined with a fluorescence Nikon eclipse E600 microscope.

qPCR analysis

Mice were killed by decapitation, hippocampi were homogenized in trizol, RNA was isolated (20), and cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using the A_{2A}R primers 5'-TAGCCCTGTGACTGAGTGCATG and 5'-GCTGCTGACCTAGAAGTGG and the GAPDH primers 5'-TGGTCCAGGGTTTCTTACTCC and 5'-

AGGTTGTCTCCTGCGACTTCA as the internal control in a realplex⁴ thermocycler (Eppendorf). The data were analyzed as described previously (20).

Behavioral evaluation

Two cohorts of mice (the ‘forebrain’ cohort comprising 17 fb-A_{2A}R KO mice and 13 fb-WT mice, and the ‘striatal’ cohort comprising 17 st-A_{2A}R KO mice and 17 st-WT mice) were used. A total of eleven tests were conducted using a within-subjects approach in the following chronologic order to minimize transfer effects, as described previously (17): (1) elevated plus maze, (2) accelerating rotarod, (3) open field, (4) Y-maze, (5) acoustic startle response, (6) prepulse inhibition, (7) Pavlovian fear conditioning, (8–11) water maze (visible, working and reference memory, reversal). Here, we report the results derived from the (i) elevated plus maze test of anxiety, (ii) spontaneous acoustic startle response, and (iii) Pavlovian fear conditioning. Another cohort with 9 fb-A_{2A}R KO and 10 fb-WT mice was used to evaluate anxiety in the open field test. Additionally, we used floxed-A_{2A}R mice intra-hippocampally injected with either AAV5-CMV-Cre-GFP (+AAV-CRE, n=7) or AAV5-CMV-GFP (control group, +AAV-CTR, n=7) to evaluate Pavlovian fear conditioning.

Anxiety

Anxiety was assessed using the elevated plus maze (21). While the total distance provided a measure of general locomotor activity, the reluctance to venture into the open arms (*i.e.*, percent time spent in and percent entries into the open arms relative to all arms) comprised the main measures of anxiety. The open field test was also used to evaluate anxiety, measuring the relative time spent in and entries into the central *versus* peripheral arenas of the activity box (22).

Associative learning: Pavlovian conditioned freezing

The apparatus consisted of two different sets of conditioning chambers to provide two distinct contexts (23). The operant chambers (context A) were from Coulbourn Instruments (Allentown, PA) and contained a grid floor to apply electric shocks (the unconditioned stimulus, US). The second set of chambers (context B) was transparent cylindrical Plexiglas enclosures resting on a white plastic floor. The conditioned stimulus (CS) was an 86 dB_A tone. The experiment consisted of three phases: conditioning, context test, and CS (tone) test. Conditioning was conducted in context A by presenting three discrete paired CS-US trials. Each trial began with a 30-s tone CS followed by delivery of a 1-s foot-shock US set at 0.26 mA. Each trial was preceded and followed by a 180-s inter-trial interval. Mice were then returned to the home cage until the context freezing test on the next day when mice were again exposed to context A for 8 min in the absence of any discrete stimulus. On the third day, conditioned freezing to the tone CS was assessed in the neutral context B. Following a 120-s acclimatization period, the tone CS was turned on for 8 min. This CS test procedure was repeated over the next three days to measure extinction of the conditioned freezing response.

In the viral-mediated hippocampal A_{2A}R knockdown experiment, we used a similar protocol with a Gemini system (San Diego Instruments, San Diego, CA). The operant chamber

(context A) contained a grid floor to apply electric shocks, and the second chamber (context B) consisted of a brown plastic floor and striped walls. CS extinction was not tested.

Acoustic startle response

The acoustic startle response was examined to provide a measure of emotional reactivity. Whole-body startle response to a sudden acoustic white-noise stimulus was measured using sound-attenuated startle chambers (San Diego Instruments, San Diego, CA) (24). A test session lasted 30 min, consisting of 106 discrete single stimulus trials presented in a pseudorandom order with a variable inter-trial interval (mean=15 s). The stimulus varied among ten possible intensities (69–120 dB_A) and two durations: 20 or 40 ms. All stimuli were in the form of a sudden elevation in broadband white noise with a rise time of 0.2–1 ms and were presented once in every successive block of 20 trials. There were five such test blocks. Prior to the 100 test trials, mice were presented with six consecutive 120-dB_A trials to allow the startle response to stabilize.

Determination of total BDNF levels in brain regions

The striatum, cerebral cortex, and hippocampus were dissected and stored at –80°C. BDNF was extracted as described (25). Total BDNF levels were determined using an ELISA assay (BDNF Emax from Promega, WI) and are presented as ng/g wet weight tissue. The standard curve was linear between 2.5 and 250 ng of BDNF.

Statistical analysis

Data were analyzed using parametric analysis of variance (ANOVA) or Student's *t*-test with genotype and sex as between-subject factors when appropriate. Within-subjects factors (bins, days, arms) were also included for repeated measures analyses when appropriate. *Post-hoc* pair-wise comparisons were performed to facilitate data interpretation when appropriate.

RESULTS

1. Reduced anxiety-related behaviors in fb-A_{2A}R KO but not in st-A_{2A}R KO mice

Striatal-selective A_{2A}R deletion did not influence anxiety-related behavior, with both st-WT and st-A_{2A}R KO mice showing indistinguishable behavior patterns in the elevated plus maze [*F*'s<1] (Figures 1A,B). However, A_{2A}R deletion in the entire forebrain reduced anxiety-related behavior in the elevated plus maze (Figures 1D,E) and in the open field paradigm (Figures 1G,H). Thus, fb-A_{2A}R KO mice spent a greater proportion of time in (Figure 1D) and entered more into (Figure 1E) the open arms than did fb-WT mice: ANOVA showed a genotype effect on both the percent time spent in [*F*_(1,26)=7.58, *p*<0.05] and the percent frequency of entries into [*F*_(1,26)=5.23, *p*<0.05] the open arms. This is likely independent of the locomotor pattern since an independent analysis of the total distance traveled failed to yield any genotypic difference in the st-A_{2A}R KO [*F*_(1,30)=1.87, *p*=0.18] (Figure 1C) or fb-A_{2A}R KO [*F*_(1,26)=2.42, *p*=0.13] (Figure 1F) cohorts. A separate cohort of fb-A_{2A}R KO mice also displayed an anxiolytic profile in an open field test, with more time spent in [*t*=2.84, *df*=15, *p*<0.05] and more entries into [*t*=2.48, *df*=17, *p*<0.05] the center arena compared to fb-WT mice (Figures 1G,H), despite exhibiting comparable total open field

locomotion [$t=1.20$, $df=17$, $p=0.25$] (Figure 1I). These results suggest that the suppression of extra-striatal hippocampal and/or cortical A_{2A}Rs but not striatal A_{2A}Rs may reduce anxiety.

2. Increased acoustic startle response in fb-A_{2A}R KO but not in st-A_{2A}R KO mice

Fb-A_{2A}R KO mice displayed higher startle responses to higher intensity stimuli compared to fb-A_{2A}R WT mice (Figure 2A). ANOVA revealed an effect of stimulus intensity [$F_{(9,234)}=162.26$, $p<0.0001$] and a genotype $\times$ stimulus intensity interaction [$F_{(9,234)}=2.47$, $p=0.01$], but no genotype effect [$p=0.22$]. Although all mice gradually responded more to startle stimuli of increasing intensity, irrespective of stimulus duration [$p=0.99$], *post-hoc* comparisons revealed that fb-A_{2A}R KO mice startled more to the two most intense stimuli (110 and 120 dB_A) relative to fb-WT mice [p 's <0.001]. This increased startle response in fb-A_{2A}R KO, but not in st-A_{2A}R KO (Figure 2B), suggests that suppression of extra-striatal hippocampal and/or cortical A_{2A}Rs but not striatal A_{2A}Rs heightens startle reactivity.

3. Effects of A_{2A}R deletion in the striatum or in the entire forebrain on fear conditioning

(a) Preserved conditioned fear acquisition (Conditioning, Day 1)—Forebrain or striatal A_{2A}R deletion did not affect shock responsiveness or perception and also did not alter the formation of the CS-US association or the execution of the conditioned freezing response (Figures 3A,E and Supplementary Materials).

(b) Enhanced context fear conditioning in st-A_{2A}R KO mice (Context test, Day 2)—Re-exposure to the same context one day after conditioning in the absence of the tone CS or shock-US induced a freezing behavior in all mice that increased over bins (Figures 3B,F). The relatively weak (10% of freezing time) magnitude of the context fear conditioned response in WT mice offers the opportunity for detecting a potentially enhanced fear response in st-A_{2A}R KO since inactivation of striatal A_{2A}Rs is associated with a pro-cognitive effect (17). Indeed, ANOVA of the percent time freezing revealed only a bins effect [$F_{(3,78)}=12.97$, $p<0.0001$] without any genotype-dependent effect in fb-A_{2A}R KO mice (Figure 3F), whereas for st-A_{2A}R KO mice there was an effect of genotype [$F_{(1,30)}=21.44$, $p<0.001$], bins [$F_{(3,90)}=8.89$, $p<0.0001$], and their interaction [$F_{(3,90)}=5.02$, $p<0.01$] (Figure 3B). This indicates that context freezing was enhanced by striatal A_{2A}R deletion, an effect that was abrogated by the additional deletion of extra-striatal A_{2A}Rs in the forebrain. This suggests that A_{2A}Rs in striatal neurons *versus* A_{2A}Rs in the rest of the forebrain (including hippocampus or cortex) modified context conditioned freezing in opposite directions. Lastly, ANCOVA using the total distance traveled during the open field test as covariate (see Supplemental Materials and Supplemental Figures 1A,B) showed that the enhanced conditioned freezing phenotype of st-A_{2A}R KO mice was not attributable to hypo-locomotion (26).

(c) Impaired tone fear conditioning in fb-A_{2A}R KO mice but enhanced tone fear conditioning in st-A_{2A}R KO mice (Tone CS test, Day 3)—The next day, mice were placed in a novel context and presented with the tone CS. While this elicited freezing in all groups of mice, the freezing response was significantly reduced in fb-A_{2A}R KO mice and enhanced in st-A_{2A}R KO mice (Figures 3C,G). ANOVA in fb-A_{2A}R KO and fb-WT mice revealed a bins effect [$F_{(4,104)}=23.17$, $p<0.0001$] and genotype $\times$ bins interaction

[$F_{(4,104)}=3.33$, $p<0.05$]. *Post-hoc* comparisons revealed that the different levels of freezing emerged in the last six minutes of testing [p 's <0.005] (Figure 3G). ANOVA in st-A_{2A}R KO and st-WT mice found effects of genotype [$F_{(1,30)}=17.94$, $p<0.0001$], bins [$F_{(4,120)}=34.79$, $p<0.0001$], and their interaction [$F_{(4,120)}=7.53$, $p<0.0001$] (Figure 3C). These data suggest that tone conditioned freezing is potentiated in st-A_{2A}R KO mice but attenuated in fb-A_{2A}R KO mice. ANCOVA using either the percent time spent in the open arms on the elevated plus maze or the startle response as covariate (Supplemental Materials) showed that the reduction in CS-induced freezing observed in fb-A_{2A}R KO mice was not solely attributable to changes in general emotional dysfunction but perhaps to another, possibly mnemonic, process.

(d) Normal progression of tone fear extinction (Tone CS extinction test, Days 3–6)—Extinction of the conditioned freezing response was examined by repeated presentation of the tone CS in the same neutral context over four consecutive days. This led to a similar reduced conditioned freezing response in both cohorts (Figures 3D,H). ANOVA of the percent time freezing confirmed this impression in both the forebrain [Days: $F_{(3,78)}=2.73$, $p<0.05$; Genotype $\times$ Days interaction: $F<1$] and striatal [Days: $F_{(3,90)}=15.99$, $p<0.0001$; Genotype $\times$ Days interaction: $F_{(3,90)}=1.21$, $p=0.31$] cohorts, thereby indicating that fear extinction was not affected in fb-A_{2A}R KO or st-A_{2A}R KO mice.

4. Intra-hippocampal injection of AAV-Cre into floxed-A_{2A}R mice attenuated context (but not tone) fear conditioning

Fluorescence GFP histology confirmed that the intra-hippocampal AAV5-CMV-GFP injection was largely restricted to the hippocampus (Figure 4A), and qPCR analysis demonstrated reduced A_{2A}R mRNA expression in the hippocampus of floxed-A_{2A}R mice injected with AAV5-CMV-Cre-GFP (Figure 4B). During the conditioning phase, AAV-CRE and AAV-CTR groups similarly acquired fear conditioning [ANOVA: Treatment: $F_{(1,36)}=0.33$, $p=0.57$; Trials: $F_{(2,36)}=39.66$, $p<0.0001$] (Figure 4C). During context conditioning the next day, the AAV-CRE group displayed significantly weaker fear responses compared to the AAV-CTR group [ANOVA: Treatment: $F_{(1,40)}=29.62$, $p<0.0001$; Bins: $F_{(3,40)}=0.24$, $p=0.86$; Treatment $\times$ Bins interaction: $F_{(3,40)}=0.24$, $p=0.87$] (Figure 4D). However, tone-induced fear responses indistinguishable between groups [ANOVA: Treatment: $F_{(1,48)}=0.38$, $p=0.54$] (Figure 4E). Thus, deletion of A_{2A}Rs restricted to the hippocampus by local AAV5-CMV-Cre-GFP infusion selectively reduced context (but not tone) fear conditioning.

5. Altered fear and anxiety-like behaviors in fb-A_{2A}R KO mice are associated with a selective reduction of BDNF levels in hippocampus

Since A_{2A}Rs control BDNF levels and can transactivate their TrkB receptors, and since BDNF levels in specific brain regions are coupled with aversive memory (27), we hypothesized that the A_{2A}R-dependent modulation of fear and anxiety behaviors might be associated with altered forebrain BDNF levels. Indeed, BDNF levels were reduced in the hippocampus but not in the striatum or motor cortex in fb-A_{2A}R KO mice compared to their WT littermates [t-test for hippocampus: $t=3.03$, $df=10$, $p<0.05$] (Figures 5A–F). No changes in BDNF levels were detected in st-A_{2A}R KO mice compared to their WT littermates.

DISCUSSION

Striatopallidal A_{2A}Rs inhibit Pavlovian fear conditioning

This is the first study, to the best of our knowledge, showing that striatal A_{2A}Rs regulate Pavlovian learned fear. Selective deletion of striatal A_{2A}Rs enhanced the conditioned freezing response to the tone CS and to the shocked context without appreciably altering the acquisition or extinction of the conditioned response. Importantly, these enhanced responses of st-A_{2A}R KO mice cannot be attributed to general emotional hyper-reactivity, abnormal fear processing, or enhanced shock detection/reactivity, and the effect remained when the confounding hypolocomotor activity effect was statistically controlled. Since we excluded differences in extinction, the stronger conditioned freezing response in st-A_{2A}R KO mice may reflect an enhanced learned fear response involving enhanced mnemonic processes, which might indicate a strong memory trace of the learned stimulus- and context-shock associations. This notion is consistent with the pro-cognitive effects seen after genetic and pharmacological inactivation of A_{2A}Rs in enhancing working memory (19, 28) and reversing age-related and pathologic memory loss (8, 9). This striatal A_{2A}R-mediated inhibition may involve the well-documented striatal D₂R-A_{2A}R antagonism, since bolstering striatal dopamine activity, through intra-accumbal injection of amphetamine (29) or a D₂R agonist (30) enhances learned fear. A similar mechanism might also underlie the inhibitory control by the activation of striatopallidal neurons over drug reward behavior (31, 32) and psychomotor sensitization (33). Our finding that striatopallidal A_{2A}Rs exert an inhibitory control over fear conditioning fits in the emerging concept that the striatopallidal pathway exerts a broad inhibitory effect on diverse types of behavior, suggesting that the striatopallidal indirect pathway may function as a “break” mechanism in the control of behavioral responses. However, this interpretation is not consistent with a recent report that selective inhibition of neurotransmission from the striatopallidal neurons attenuated fear conditioning (34). Additional studies are required to clarify this issue.

Inactivation of extra-striatal A_{2A}Rs in the forebrain counters the actions of striatal A_{2A}Rs on Pavlovian fear conditioning

When A_{2A}R deletion was extended beyond the striatum to cortical and hippocampal regions, the impact of striatal A_{2A}R inactivation on context and tone fear conditioning was normalized or reversed. Indeed, forebrain A_{2A}R elimination impaired, rather than enhanced, tone fear conditioning though sparing normal context freezing. Although the genetic backgrounds differ between forebrain and striatum KO lines, which may account for the difference in freezing levels observed between fb-WT and st-WT mice, comparison of each conditional KO line against its respective littermate WT controls clearly demonstrates the opposite impacts on fear conditioning in the two KO lines.

These opposite phenotypes may be attributable to hippocampal, cortical, or amygdalar A_{2A}Rs. In particular, AAV5-Cre experiments revealed the role of hippocampal A_{2A}Rs in controlling context (but not tone) fear conditioning. Conversely, cortical A_{2A}Rs may enhance tone fear conditioning since inactivating perirhinal, parietal, or cingulate cortical activity selectively impairs fear tone conditioning (35–37). A_{2A}Rs in the amygdala also might contribute given the role of this brain region in fear responses. Additional analyses

with focal deletion of prefrontal cortical and amygdalar A_{2A}Rs by AAV-Cre may identify the forebrain structure(s) where the loss of A_{2A}R overrides the opposite impact of striatal A_{2A}R deletion.

Extra-striatal A_{2A}Rs in the forebrain attenuate anxiety-like behavior but increase startle response

When A_{2A}R deletion was restricted to the striatum, no change in anxiety-like behavior was observed. When the deletion was extended to the cortex and hippocampus in fb-A_{2A}R KO mice, anxiolytic behavior in the elevated plus maze and the open field was observed. This finding is consistent with the hippocampus and cortex as relevant regions controlling anxiety (38–40). However, anxiolysis seen following forebrain A_{2A}R constitutive inactivation contrasts with the anxiogenic effect of acute caffeine (a non-selective adenosine receptor antagonist) (41) and with genetic association studies between the A_{2A}R gene and panic disorder (42–44). A_{2A}Rs in brain regions beyond the forebrain need to be examined to clarify this issue.

It is well known that a fearful state or heightened anxiety can potentiate startle reactions. The acoustic startle response was selectively increased only at the two highest stimulus intensities in fb-A_{2A}R KO mice while no effect was found in st-A_{2A}R KO mice. Such an intensity-dependent enhancement most likely reflects changes in the maximal startle response rather than an anxiogenic phenotype (45–47). The lack of a startle phenotype in st-A_{2A}R KO mice is also consistent with the null-effect of intra-accumbal infusions of A_{2A}R ligands on startle responses (48, 49). Notably, forebrain A_{2A}R-dependent regulation of acoustic startle and elevated plus maze behaviors resemble the behavioral phenotype induced by septal lesions, which produced an ‘anxiolytic’ effect in the elevated plus maze and ‘anxiogenic’ stronger acoustic startle responses (e.g., 50). Thus, forebrain A_{2A}R deletion may induce these seemingly paradoxical phenotypes via an effect on the septum.

A_{2A}R-dependent control of conditioned and unconditioned fear behavior in extra-striatal forebrain is associated with selective alteration of BDNF levels

BDNF modulation of synaptic plasticity (51, 52) may underlie the BDNF-mediated control of conditioned fear and anxiety. Clearly, there are different pools of BDNF in the hippocampus, resulting from different transcripts expressed in different cellular and subcellular compartments, possibly fulfilling different functional roles. It is therefore likely and understandable that the ablation of the BDNF gene (eliminating all BDNF) should produce an impact different from the putative selective reduction of a particular pool of BDNF. Recently, several studies have reported that BDNF levels in the dorsal hippocampus are associated with increased emotional reactivity (53), context fear conditioning (54, 55), anxiety (55–57), and altered behavior in PTSD animal models (58, 59). Notably, the effect of BDNF on hippocampal synaptic plasticity requires A_{2A}R activation (7) whereas global A_{2A}R inactivation decreases BDNF levels (60), reduces the activation of the major BDNF receptor (TrkB), and impairs BDNF-dependent-LTP in the hippocampus (61, 62). Thus, hippocampal A_{2A}R deletion might impair fear conditioning by interfering with the BDNF-TrkB pathway. This observation is consistent with the present finding that a selective BDNF reduction in the hippocampus is associated with reduced tone fear conditioning and anxiety

behavior in fb-A_{2A}R KO (but not in st-A_{2A}R KO) mice. However, the differential modifications of context fear learning upon elimination of A_{2A}Rs in the forebrain (fb-A_{2A}R KO) and selectively in the hippocampus (by viral approach) clearly indicates that the reduction of BDNF in the hippocampus of fb-A_{2A}R KO mice is likely not causally related to increased tone freezing. Other mechanisms apart from the control of hippocampal BDNF levels are involved in extra-striatal A_{2A}R-control of fear responses. Future studies with rescue experiments (*e.g.* augmenting BDNF expression) in the hippocampus and additional related brain regions like nucleus accumbens, prefrontal cortex, and lateral and central amygdala in fb-A_{2A}R KO mice would be required to establish a causal link between the reduction in BDNF in distinct forebrain regions and the fear conditioning phenotype in fb-A_{2A}R KO mice.

In summary, we have demonstrated distinctive roles of striatal and extra-striatal A_{2A}Rs in the control of conditioned and unconditioned fear behavior. Inactivation of striatal A_{2A}Rs facilitates Pavlovian fear conditioning while inactivation of extra-striatal A_{2A}Rs in the forebrain inhibits fear conditioning and also affects anxiety-related behavior. Our work also identifies the previously under-appreciated A_{2A}Rs in cortex and hippocampus as contributing regulators of conditioned fear, anxiety, and startle response with a parallel reduction of hippocampal BDNF. Enhanced fear conditioning and increased startle response are important behavioral elements in animal models for PTSD (61, 62). We demonstrate that selective deletion of A_{2A}Rs in the forebrain yields alterations in all three of the defining features of PTSD in rodent models (*i.e.*, fear conditioning, startle response, and anxiety). Thus, targeting forebrain A_{2A}Rs may provide a novel therapeutic strategy for PTSD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by grants from NIH (R01MH083973, RO1NS48995, RO1NS41083-07, RO1DA19362), the Department of Defense (W81XWH-071-1-0012, DARPA-BAA-09-68), and FCT (PTDC/SAU-NEU/108668/2008, PTDC/SAU-NSC/122254/2010).

REFERENCES

1. Schiffmann SN, Fisone G, Moresco R, Cunha RA, Ferre S. Adenosine A_{2A} receptors and basal ganglia physiology. *Progress in neurobiology*. 2007; 83:277–292. [PubMed: 17646043]
2. Svenningsson P, Le Moine C, Fisone G, Fredholm BB. Distribution, biochemistry and function of striatal adenosine A_{2A} receptors. *Progress in neurobiology*. 1999; 59:355–396. [PubMed: 10501634]
3. Rebola N, Lujan R, Cunha RA, Mulle C. Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron*. 2008; 57:121–134. [PubMed: 18184569]
4. Costenla AR, Diogenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J, et al. Enhanced role of adenosine A_{2A} receptors in the modulation of LTP in the rat hippocampus upon ageing. *The European journal of neuroscience*. 34:12–21. [PubMed: 21615561]
5. d'Alcantara P, Ledent C, Swillens S, Schiffmann SN. Inactivation of adenosine A_{2A} receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. *Neuroscience*. 2001; 107:455–464. [PubMed: 11719000]

6. Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnong N, Bendor J, et al. FGF acts as a co-transmitter through adenosine A(2A) receptor to regulate synaptic plasticity. *Nature neuroscience*. 2008; 11:1402–1409.
7. Sebastiao AM, Ribeiro JA. Triggering neurotrophic factor actions through adenosine A2A receptor activation: implications for neuroprotection. *British journal of pharmacology*. 2009; 158:15–22. [PubMed: 19508402]
8. Cunha RA, Agostinho PM. Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. *J Alzheimers Dis*. 2010; 20(Suppl 1):S95–S116. [PubMed: 20182043]
9. Takahashi RN, Pamplona FA, Prediger RD. Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. *Front Biosci*. 2008; 13:2614–2632. [PubMed: 17981738]
10. Dall'igna OP, Fett P, Gomes MW, Souza DO, Cunha RA, Lara DR. Caffeine and adenosine A(2a) receptor antagonists prevent beta-amyloid (25–35)-induced cognitive deficits in mice. *Experimental neurology*. 2007; 203:241–245. [PubMed: 17007839]
11. Kopf SR, Melani A, Pedata F, Pepeu G. Adenosine and memory storage: effect of A(1) and A(2) receptor antagonists. *Psychopharmacology (Berl)*. 1999; 146:214–219. [PubMed: 10525758]
12. Pereira GS, Rossato JJ, Sarkis JJ, Cammarota M, Bonan CD, Izquierdo I. Activation of adenosine receptors in the posterior cingulate cortex impairs memory retrieval in the rat. *Neurobiol Learn Mem*. 2005; 83:217–223. [PubMed: 15820857]
13. El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. *Psychopharmacology (Berl)*. 2000; 148:153–163. 150.htm. [PubMed: 10663430]
14. Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, et al. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature*. 1997; 388:674–678. [PubMed: 9262401]
15. Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY, et al. A critical role of the adenosine A2A receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A2A receptor knockouts. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008; 28:2970–2975. [PubMed: 18354001]
16. Bastia E, Xu YH, Scibelli AC, Day YJ, Linden J, Chen JF, et al. A crucial role for forebrain adenosine A(2A) receptors in amphetamine sensitization. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2005; 30:891–900. [PubMed: 15602504]
17. Wei CJ, Singer P, Coelho J, Boison D, Feldon J, Yee BK, et al. Selective inactivation of adenosine A(2A) receptors in striatal neurons enhances working memory and reversal learning. *Learn Mem*. 2011; 18:459–474. [PubMed: 21693634]
18. Yu L, Shen HY, Coelho JE, Araujo IM, Huang QY, Day YJ, et al. Adenosine A2A receptor antagonists exert motor and neuroprotective effects by distinct cellular mechanisms. *Ann Neurol*. 2008; 63:338–346. [PubMed: 18300283]
19. Wei CJ, Singer P, Coelho J, Boison D, Feldon J, Yee BK, et al. Selective inactivation of adenosine A2A receptors in striatal neurons enhances working memory and reversal learning. *Learn Mem*. 2011; 18:459–474. [PubMed: 21693634]
20. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols*. 2008; 3:1101–1108.
21. Hagenbuch N, Feldon J, Yee BK. Use of the elevated plus-maze test with opaque or transparent walls in the detection of mouse strain differences and the anxiolytic effects of diazepam. *Behavioural pharmacology*. 2006; 17:31–41. [PubMed: 16377961]
22. Pietropaolo S, Mintz M, Feldon J, Yee BK. The behavioral sequela following the prevention of home-cage grid-climbing activity in C57BL/6 mice. *Behav Neurosci*. 2007; 121:345–355. [PubMed: 17469924]

23. Yee BK, Singer P, Chen JF, Feldon J, Boison D. Transgenic overexpression of adenosine kinase in brain leads to multiple learning impairments and altered sensitivity to psychomimetic drugs. *The European journal of neuroscience*. 2007; 26:3237–3252. [PubMed: 18005073]
24. Yee BK, Chang DL, Feldon J. The Effects of dizocilpine and phencyclidine on prepulse inhibition of the acoustic startle reflex and on prepulse-elicited reactivity in C57BL6 mice. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2004; 29:1865–1877. [PubMed: 15127085]
25. Szapacs ME, Mathews TA, Tessarollo L, Ernest Lyons W, Mamounas LA, Andrews AM. Exploring the relationship between serotonin and brain-derived neurotrophic factor: analysis of BDNF protein and extraneuronal 5-HT in mice with reduced serotonin transporter or BDNF expression. *J Neurosci Methods*. 2004; 140:81–92. [PubMed: 15589338]
26. Singer P, Zhang CC, Boison D, Yee BK. Dysregulation of brain adenosine is detrimental to the expression of conditioned freezing but not general Pavlovian learning. *Pharmacology, biochemistry, and behavior*. 2013
27. Ma L, Wang DD, Zhang TY, Yu H, Wang Y, Huang SH, et al. Region-specific involvement of BDNF secretion and synthesis in conditioned taste aversion memory formation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2011; 31:2079–2090. [PubMed: 21307245]
28. Zhou SJ, Zhu ME, Shu D, Du XP, Song XH, Wang XT, et al. Preferential enhancement of working memory in mice lacking adenosine A(2A) receptors. *Brain Res*. 2009; 1303:74–83. [PubMed: 19785999]
29. White NM, Salinas JA. Mnemonic functions of dorsal striatum and hippocampus in aversive conditioning. *Behav Brain Res*. 2003; 142:99–107. [PubMed: 12798270]
30. White NM, Viaud M. Localized intracaudate dopamine D2 receptor activation during the post-training period improves memory for visual or olfactory conditioned emotional responses in rats. *Behav Neural Biol*. 1991; 55:255–269. [PubMed: 1676259]
31. Durieux PF, Schiffmann SN, de Kerchove d'Exaerde A. Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions. *EMBO J*. 2012; 31:640–653. [PubMed: 22068054]
32. Lobo MK, Covington HE 3rd, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, et al. Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. *Science*. 2010; 330:385–390. [PubMed: 20947769]
33. Bateup HS, Santini E, Shen W, Birnbaum S, Valjent E, Surmeier DJ, et al. Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:14845–14850. [PubMed: 20682746]
34. Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S. Distinct roles of synaptic transmission in direct and indirect striatal pathways to reward and aversive behavior. *Neuron*. 2010; 66:896–907. [PubMed: 20620875]
35. Biedenkapp JC, Rudy JW. Hippocampal and extrahippocampal systems compete for control of contextual fear: role of ventral subiculum and amygdala. *Learn Mem*. 2009; 16:38–45. [PubMed: 19117915]
36. Bissiere S, Plachta N, Hoyer D, McAllister KH, Olpe HR, Grace AA, et al. The rostral anterior cingulate cortex modulates the efficiency of amygdala-dependent fear learning. *Biological psychiatry*. 2008; 63:821–831. [PubMed: 18155183]
37. Sacchetti B, Baldi E, Lorenzini CA, Bucherelli C. Differential contribution of some cortical sites to the formation of memory traces supporting fear conditioning. *Exp Brain Res*. 2002; 146:223–232. [PubMed: 12195524]
38. McHugh SB, Fillenz M, Lowry JP, Rawlins JN, Bannerman DM. Brain tissue oxygen amperometry in behaving rats demonstrates functional dissociation of dorsal and ventral hippocampus during spatial processing and anxiety. *The European journal of neuroscience*. 2005; 17:3322–3327. [PubMed: 21105915]
39. Adhikari A, Topiwala MA, Gordon JA. Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety. *Neuron*. 2011; 69:257–269. [PubMed: 2152131]

40. Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, et al. Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev.* 2004; 28:273–283. [PubMed: 15225971]
41. Cunha RA, Ferre S, Vaugeois JM, Chen JF. Potential therapeutic interest of adenosine A2A receptors in psychiatric disorders. *Curr Pharm Des.* 2008; 14:1512–1524. [PubMed: 18537674]
42. Hamilton SP, Slager SL, De Leon AB, Heiman GA, Klein DF, Hodge SE, et al. Evidence for genetic linkage between a polymorphism in the adenosine 2A receptor and panic disorder. *Neuropsychopharmacology.* 2004; 29:558–565. [PubMed: 14666117]
43. Hohoff C, Mullings EL, Heatherley SV, Freitag CM, Neumann LC, Domschke K, et al. Adenosine A(2A) receptor gene: evidence for association of risk variants with panic disorder and anxious personality. *J Psychiatr Res.* 2010; 44:930–937. [PubMed: 20334879]
44. Deckert J, Nothen MM, Franke P, Delmo C, Fritze J, Knapp M, et al. Systematic mutation screening and association study of the A1 and A2a adenosine receptor genes in panic disorder suggest a contribution of the A2a gene to the development of disease. *Molecular psychiatry.* 1998; 3:81–85. [PubMed: 9491818]
45. Hince DA, Martin-Iverson MT. Differences in prepulse inhibition (PPI) between Wistar and Sprague-Dawley rats clarified by a new method of PPI standardization. *Behavioral neuroscience.* 2005; 119:66–77. [PubMed: 15727513]
46. Martin-Iverson MT, Stevenson KN. Apomorphine effects on emotional modulation of the startle reflex in rats. *Psychopharmacology (Berl).* 2005; 181:60–70. [PubMed: 15772861]
47. Stoddart CW, Noonan J, Martin-Iverson MT. Stimulus quality affects expression of the acoustic startle response and prepulse inhibition in mice. *Behavioral neuroscience.* 2008; 122:516–526. [PubMed: 18513122]
48. Hauber W, Koch M. Adenosine A2a receptors in the nucleus accumbens modulate prepulse inhibition of the startle response. *Neuroreport.* 1997; 8:1515–1518. [PubMed: 9172165]
49. Nagel J, Schladebach H, Koch M, Schwienbacher I, Muller CE, Hauber W. Effects of an adenosine A2A receptor blockade in the nucleus accumbens on locomotion, feeding, and prepulse inhibition in rats. *Synapse.* 2003; 49:279–286. [PubMed: 12827647]
50. Decker MW, Curzon P, Brioni JD. Influence of separate and combined septal and amygdala lesions on memory, acoustic startle, anxiety, and locomotor activity in rats. *Neurobiology of learning and memory.* 1995; 64:156–168. [PubMed: 7582824]
51. Poo MM. Neurotrophins as synaptic modulators. *Nat Rev Neurosci.* 2001; 2:24–32. [PubMed: 11253356]
52. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci.* 2003; 4:299–309. [PubMed: 12671646]
53. Heldt SA, Stanek L, Chhatwal JP, Ressler KJ. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Molecular psychiatry.* 2007; 12:656–670. [PubMed: 17264839]
54. Bozdagi O, Rich E, Tronel S, Sadahiro M, Patterson K, Shapiro ML, et al. The neurotrophin-inducible gene *Vgf* regulates hippocampal function and behavior through a brain-derived neurotrophic factor-dependent mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2008; 28:9857–9869. [PubMed: 18815270]
55. Yee BK, Zhu SW, Mohammed AH, Feldon J. Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice. *J Neural Transm.* 2007; 114:431–444. [PubMed: 16897598]
56. Texel SJ, Camandola S, Ladenheim B, Rothman SM, Mughal MR, Unger EL, et al. Ceruloplasmin deficiency results in an anxiety phenotype involving deficits in hippocampal iron, serotonin, and BDNF. *Journal of neurochemistry.* 120:125–134. [PubMed: 22035068]
57. Aso E, Ozaita A, Valdizan EM, Ledent C, Pazos A, Maldonado R, et al. BDNF impairment in the hippocampus is related to enhanced despair behavior in CB1 knockout mice. *Journal of neurochemistry.* 2008; 105:565–572. [PubMed: 18047561]
58. Takei S, Morinobu S, Yamamoto S, Fuchikami M, Matsumoto T, Yamawaki S. Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. *J Psychiatr Res.* 45:460–468. [PubMed: 20863519]

59. Kozlovsky N, Matar MA, Kaplan Z, Kotler M, Zohar J, Cohen H. Long-term down-regulation of BDNF mRNA in rat hippocampal CA1 subregion correlates with PTSD-like behavioural stress response. *Int J Neuropsychopharmacol*. 2007; 10:741–758. [PubMed: 17291374]
60. Tebano MT, Martire A, Potenza RL, Gro C, Pepponi R, Armida M, et al. Adenosine A(2A) receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus. *Journal of neurochemistry*. 2008; 104:279–286. [PubMed: 18005343]
61. Lee FS, Chao MV. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:3555–3560. [PubMed: 11248116]
62. Assaife-Lopes N, Sousa VC, Pereira DB, Ribeiro JA, Chao MV, Sebastiao AM. Activation of adenosine A2A receptors induces TrkB translocation and increases BDNF-mediated phospho-TrkB localization in lipid rafts: implications for neuromodulation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 30:8468–8480. [PubMed: 20573894]

Figure 1. Reduced anxiety-like behavior on the elevated plus maze and open field tests in fb-A_{2A}R KO but not in st-A_{2A}R KO mice

To measure unlearned emotional or fear-related processing, anxiety-related behavior was evaluated in the elevated plus maze (EPM) for 5 min in naïve st-A_{2A}R KO and fb-A_{2A}R KO mice (A–F). Further evaluation in the open field for 8 min in a separate cohort of naïve fb-A_{2A}R KO mice was performed (G–I). **EPM (st-A_{2A}R KO and fb-A_{2A}R KO):** (A) St-A_{2A}R KO and st-WT mice spent a similar percentage of time in the open arms of the maze. (B) Likewise, st-A_{2A}R KO and st-WT mice entered into the open arms of the maze at a similar frequency compared to the closed arms of the maze. (C) St-A_{2A}R KO and st-WT mice traveled a similar distance on the maze. (D) Fb-A_{2A}R KO mice spent a significantly greater proportion of time in the open arms relative to the closed arms, suggesting reduced anxiety compared to fb-WT mice. (E) Fb-A_{2A}R KO mice also entered into the open arms proportionally more than the closed arms compared to fb-WT mice, further supporting reduced anxiety-like behavior in fb-A_{2A}R KO mice. (F) Fb-A_{2A}R KO and fb-WT mice traveled a similar distance on the maze. **Open field (fb-A_{2A}R KO):** Fb-A_{2A}R KO mice also showed reduced anxiety-related behavior in the open field. (G) Fb-A_{2A}R KO mice spent more time in the center arena compared to fb-WT mice. (H) Fb-A_{2A}R KO mice also entered into the center arena more frequently compared to fb-WT mice. (I) Fb-A_{2A}R KO and fb-WT

mice traveled a similar distance in the open field. Data are mean $\pm$ SEM. * $p < 0.05$, fb-A_{2A}R KO vs fb-WT. EPM: st-A_{2A}R KO, n=17; st-WT, n=17; fb-A_{2A}R KO, n=17; fb-WT, n=13. Open field: fb-A_{2A}R KO, n=9; fb-WT, n=10.

Figure 2. Increased startle response in fb-A_{2A}R KO but not in st-A_{2A}R KO mice

The acoustic startle response (ln-transformed) $\pm$ SEM to ten acoustic stimuli of increasing intensity is depicted (collapsed across stimuli durations of 20 and 40 ms). Startle reactivity increased monotonically as a function of stimulus intensity. **(A)** Compared to fb-WT mice, fb-A_{2A}R KO mice exhibited increased startle responses to the two highest stimulus intensities (110 and 120 dB_A). **(B)** In contrast, st-A_{2A}R KO mice and st-WT mice displayed similar sensitivity to acoustic stimuli. *** $p < 0.001$, fb-A_{2A}R KO vs fb-WT. st-A_{2A}R KO, $n=17$; st-WT, $n=17$; fb-A_{2A}R KO, $n=17$; fb-WT, $n=13$.

Figure 3. Opposite effects of selective deletion of $A_{2A}R$ s in the striatum or in the entire forebrain on fear conditioning

Conditioned freezing (% Time) during conditioning acquisition (day 1), context test (day 2), and tone conditioned stimulus (CS) tests (days 3–6) are shown. **(A, E) Conditioning:**

Freezing responses to three repeated presentations of a 30-s CS paired with an unconditioned stimulus (1-s foot-shock US) are illustrated. All mice showed a comparable increase in freezing with each successive CS-US-paired trial. **(B, F) Context Test:** Freezing responses to the conditioning context in the absence of the CS one day later are illustrated.

St- $A_{2A}R$ KO mice exhibited more pronounced freezing throughout the entire 8-min test relative to st-WT mice while fb- $A_{2A}R$ KO mice exhibited indistinguishable freezing from fb-WT mice. **(C, G) CS Test:** Freezing responses during four consecutive daily CS tests in a new context are illustrated; each test included a 2-min CS-free period followed by an 8-min CS presentation. St- $A_{2A}R$ KO mice consistently showed a marked increase in CS freezing compared to st-WT mice; by contrast, fb- $A_{2A}R$ KO mice consistently showed a reduced CS freezing response compared to fb-WT mice. **(D, H) CS Extinction:** Freezing responses (daily 2-min bin mean) to the repeated presentation of only the CS across the 4 test days are depicted. The observed reduction in freezing across days suggests extinction, which was comparable between st- $A_{2A}R$ KO, fb- $A_{2A}R$ KO mice, and their corresponding WT littermates. Data are mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.005$, st- $A_{2A}R$ KO vs st-WT or fb- $A_{2A}R$ KO vs fb-WT. st- $A_{2A}R$ KO, $n = 17$; st-WT, $n = 17$; fb- $A_{2A}R$ KO, $n = 17$; fb-WT, $n = 13$.

Figure 4. Deletion of hippocampal A_{2A}Rs by local injection of AAV5-CMV-Cre-GFP into conditional (floxed)-A_{2A}R mice attenuates context (but not tone) fear conditioning
AAV5-CMV-Cre-GFP (+AAV-CRE) or AAV5-CMV-GFP (+AAV-CTR) were injected into both hippocampi of floxed-A_{2A}R mice. Fluorescence histology and qPCR analyses were conducted to confirm selective AAV5-mediated A_{2A}R deletion in hippocampus. Fear conditioning acquisition, context and tone tests were conducted three weeks after injection. (A) Fluorescence GFP histology in the hippocampus of an AAV-CTR group mouse shows restricted infection of the hippocampus. (B) qPCR analysis of A_{2A}R mRNA in hippocampus shows reduced A_{2A}R expression in AAV-CRE mice. (C) Acquisition of fear responses was similar between the two treatment groups. (D) The context fear response was attenuated in the AAV-CRE group compared to the AAV-CTR group. (E) Tone fear conditioning was

indistinguishable between groups. Data are mean $\pm$ SEM. * $p < 0.05$, $^{***}p < 0.001$, AAV-CRE *vs* AAV-CTR. $n = 7$ per group.

Figure 5. Brain-derived neurotrophic factor (BDNF) levels are selectively reduced in the hippocampus of fb-A_{2A}R KO but not of st-A_{2A}R KO mice

The average quantification of BDNF levels in different brain regions, namely the motor cortex (A, D), hippocampus (B, E) and striatum (C, G), of fb-A_{2A}R KO and WT mice (A–C) and str-A_{2A}R KO and WT mice (D–F), are depicted. Total BDNF levels were quantified by ELISA. Data are mean ± SEM. *p<0.05 compared to the respective WT mice by Student's *t*-test. n=5–6 per group.