Regulation of Fear Responses by Striatal and Extrastriatal Adenosine A_{2A} Receptors in Forebrain

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Background: Adenosine A_{2A} receptors (A_{2A}Rs) are enriched in the striatum but are also present at lower levels in the extrastriatal 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 deleted either in the striatum (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 adeno-associated virus type 5 (AAV5)-Cre into floxed-A_{2A}R knockout mice.

Results: Selectively deleting A_{2A}Rs in the striatum increased Pavlovian fear conditioning (both context and tone) in st-A_{2A}R KO mice, but extending the deletion to the rest of the forebrain apparently spared context fear conditioning and attenuated tone fear conditioning in fb-A_{2A}R KO mice. 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 extrastriatal forebrain A_{2A}R behavioral effects were associated with reduced BDNF levels in the fb-A_{2A}R KO hippocampus.

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

Key Words: Adenosine A_{2A} receptor, anxiety, BDNF, cortex, fear conditioning, hippocampus, startle response, striatum

Conditioned fear predicts aversive events from prior experience, and its dysfunction leads to maladaptive fear responses that underlie neuropsychiatric disorders such as posttraumatic 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 extrastriatal forebrain (fb), 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 tropomyosin-related kinase B (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 pharmacologic (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 1) different contributions of distinct brain regions to emotional responses; 2) potentially different effects of A_{2A}Rs on different emotional processes such as conditioned fear and anxiety; and 3) 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 [st] versus hippocampus and cerebral cortex) exert an opposite control over psychomotor responses (15).

To molecularly dissect the striatal and extrastriatal contributions of forebrain A_{2A}Rs to emotional processes, we analyzed conditioned and innate fear responses in two conditional A_{2A}R knockout (KO) mouse 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 A_{2A}Rs in the entire forebrain or focal deletion of hippocampal A_{2A}Rs normalized or attenuated context and tone fear conditioning while also producing an anxiolytic phenotype. We conclude that striatal and extrastriatal 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 Institutional Animal Care and Use Committees 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.

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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-wild-type [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[−] $A_{2A}R^{flox/flox}$]) were also generated as previously described (15,17). Fb-$A_{2A}R$ KO mice with $A_{2A}R$ deletion extended beyond the striatum to the entire forebrain, including cerebral cortex and hippocampus, 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, respectively, and as validated by: 1) X-gal staining of a Cre-expressing Rosa26 reporter transgenic line; 2) polymerase chain reaction (PCR) analysis of Cre-mediated $A_{2A}R$ deletion; 3) $A_{2A}R$ immunohistochemistry localization; and 4) $A_{2A}R$ binding with ^{3}H-ZM241385 (15–18).

Intrahippocampal Injection of Adeno-Associated Virus Type 5-Cre into Floxed-$A_{2A}R$ Mice

Either adeno-associated virus type 5 (AAV5)-cytomegalovirus (CMV)-Cre-green fluorescent protein (GFP) (Vector BioLabs, Philadelphia, Pennsylvania; 2 μL of 1×10^{12} genome copies/mL) or its control viral vector (AAV5-CMV-GFP) were injected stereotaxically into both hippocampi of floxed-$A_{2A}R$ adult mice (anteroposterior = −2.5 mm from bregma, mediolateral = ±2.0 mm from midline, and dorsoventral = −1.5 mm from the skull surface). Mice were tested for conditioned freezing 3 weeks later.

Fluorescence Immunohistochemistry

Mice were anesthetized with Avertin (Sigma-Aldrich, St. Louis, Missouri; 2% 2,2,2-tribromoethanol and 1% amyl alcohol). Brains were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS), postfixed, and cryopreserved. Coronal sections (30 μm) (Leica Microsystems) were incubated for 1 hour in PBS containing .25% Triton X-100 (FisherBiotech, Fair Lawn, New Jersey) and 5% donkey serum (Jackson Immunoresearch, West Grove, Pennsylvania), followed by incubation with mouse anti-mouse NeuN (1:1000; Millipore, Billerica, Massachusetts) and rabbit anti-GFP (1:1000; Abcam, Cambridge, Massachusetts) antibodies overnight at 4°C. Sections were rinsed 3 × 10 minutes 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, Carlsbad, California) for 1 hour at room temperature. Sections were rinsed, mounted with Vectashield mounting medium, and examined with a fluorescence Nikon (Melville, New York) eclipse E600 microscope.

Quantitative Polymerase Chain Reaction Analysis

Mice were killed by decapitation, hippocampi were homogenized in trizol, RNA was isolated (19), and complementary DNA 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, Carlsbad, California) using the $A_{2A}R$ primers 5′-TAGCCCTGTGACTGAGTGCATG and 5′- GCTGCTGACCTAGAAGTGG and the glyceraldehyde-3-phosphate dehydrogenase primers 5′-TGGTCCAGGGTTTCTTACTCC and 5′-AGGTTGTCTCCTGCGACTTCA as the internal control in a realplex4 thermocycler (Eppendorf, Hamburg, Germany). The data were analyzed as described previously (19).

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 11 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) water maze, visible; 9) water maze, working memory; 10) water maze, reference memory; and 11) water maze, reversal. Here, we report the results derived from the elevated plus maze test of anxiety, spontaneous acoustic startle response, and 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 intrahippocampally injected with either AAV5-CMV-Cre-GFP (+AAV5-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 (20). 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 (21).

Associative Learning: Pavlovian Conditioned Freezing

The apparatus consisted of two different sets of conditioning chambers to provide two distinct contexts (22). The operant chambers (context A) were from Coulbourn Instruments (Allentown, Pennsylvania) 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-second tone CS followed by delivery of a 1-second foot-shock US set at .26 mA. Each trial was preceded and followed by a 180-second intertrial 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 minutes 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-second acclimatization period, the tone CS was turned on for 8 minutes. This CS test procedure was repeated over the next 3 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, California). 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) (23). A test session lasted 30 minutes, consisting of 106 discrete single stimulus