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Dysregulation of brain adenosine is detrimental to the expression of conditioned freezing but not general Pavlovian learning ☆

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ABSTRACT

Glutamatergic and dopaminergic neurotransmission is modulated by adenosine, whose ambient level in the brain is in turn regulated by the metabolic enzyme, adenosine kinase (ADK). Brain adenosinergic tone can therefore be effectively reduced and increased by up- and down-regulation of ADK expression, respectively. Although changes in brain ADK levels can yield multiple behavioral effects, the precise functional significance of telencephalon (neocortical and limbic structures) adenosine remains ill-defined. Among the phenotypes identified in transgenic mice with brain-wide ADK overexpression (*ADK*^{TG} mice) and reduced adenosinergic tone, working memory deficiency and potentiated response to systemic *N*-methyl-D-aspartate receptor blockade were exacerbated by the introduction of local ADK disruption (elevated adenosinergic tone) restricted to the telencephalon (*ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice). These two phenotypes, which are central to schizophrenia cognitive/negative symptoms, appear to be regulated by adenosinergic activities within and outside the telencephalon in a complementary manner. Here, we extended this unique comparison between *ADK*^{TG} mice *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice to another prominent phenotype previously documented in *ADK*^{TG} mice — namely, impaired Pavlovian conditioned freezing. We found that *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice again were associated with a more severe phenotype while sharing a similar phenotype profile. Furthermore, we qualified that this Pavlovian phenotype did not translate into a general deficiency in associative learning, since no such deficit was evident in three other (aversive and appetitive) Pavlovian learning paradigms. The present study has thus identified a hitherto unknown function of brain adenosine: the execution of conditioned freezing behavior, which is dependent on the balance of adenosinergic changes between the telencephalon and the rest of the brain.

1. Introduction

As an integrator of glutamatergic and dopaminergic signaling in the brain, the neuromodulator adenosine can exert extensive influence over behavioral outputs including cognitive performance (e.g., Boison et al., 2012; Fredholm et al., 2005; Sebastião and Ribeiro, 2009). Adenosine binds to G-protein coupled adenosine receptors, A_1 and A_{2A}, each with a distinct expression pattern in the brain, and are separately linked to specific interactions with dopamine and glutamate receptors (for reviews, see Jacobson and Gao, 2006; Ribeiro et al., 2003). The extracellular neuro-active pools of adenosine in the brain are mainly controlled by adenosine kinase (ADK), an astrocytic enzyme that catalyzes the phosphorylation of adenosine thereby driving the influx of adenosine into astrocytes through passive transporters (Boison, 2006; Etherington et al., 2009). Up-regulation of ADK facilitates adenosine clearance and therefore reduces extracellular adenosine levels as demonstrated in the *ADK*^{TG} mice, in which the endogenous ADK gene was replaced by a ubiquitin-driven ADK transgene (Fedele et al., 2005). The resulting brain-wide decrease in adenosine impaired working memory function and sensitized the motor response to systemic *N*-methyl-D-aspartate receptor (NMDAR) blockade (Singer et al., 2012; Yee et al., 2007) — phenotypes that are relevant to the negative and cognitive symptoms of schizophrenia (Boison et al., 2012). In an attempt to delineate the contribution of adenosine overexpression in the telencephalon (neocortical and limbic structures) to these phenotypes, a new mouse line was created by introducing a selective disruption of the ADK transgene into the telencephalon within the *ADK*^{TG} background. These new mutant mice (*ADK*^{TG}:*ADK*$^{Tel\text{-}def}$) had elevated extracellular adenosine throughout the cortical mantle, hippocampus and amygdala, while the rest of the brain remained as adenosine-deficient as in the original *ADK*^{TG} line (Shen et al., 2011; Singer et al., 2012). Comparison

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between the two mutants thus provides a unique contrast between up- and down-regulation of telencephalon ADK against a background of adenosine deficiency in the rest of the brain — out of which the striatum is the structure with the highest expression of adenosine receptors, especially A_{2A}Rs (e.g. Ribeiro et al., 2003; Svenningsson et al., 1999b).

The rationale was as follows: If the overexpression of ADK (deficiency of adenosine) within corticolimbic structures critically underlies the working memory and NMDAR blockade-induced phenotypes in *ADK*^{TG} mice, then reversing the changes in ADK/adenosine (i.e., disruption of ADK and elevation of adenosine) within these brain structures should yield phenotypes in the opposite direction or at least in severely attenuated form of expression. However, both phenotypes were found to be exacerbated in the *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice compared with *ADK*^{TG} mice (Singer et al., 2012). This unexpected finding has led to the conclusion that, against the backdrop of adenosine deficiency outside the telencephalon (primarily in the striatum), efficient working memory performance and the integrity of NMDAR function is sensitive to both up- and down-regulation of corticolimbic adenosinergic tone, perhaps via A_1R- and A_{2A}R-dependent mechanisms, respectively. The more severe effects of corticolimbic adenosinergic tone up-regulation might stem from a stronger striatal–cortical imbalance of adenosinergic tone in *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice. These insights gleaned from the unique comparison between *ADK*^{TG} and *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice were further tested here by examining another prominent phenotype previously identified in *ADK*^{TG} mice — namely, impaired Pavlovian conditioned freezing (Yee et al., 2007). To gauge the importance of this specific phenotype to Pavlovian learning in general, we extended the test to other associative learning paradigms, including two-way conditioned active avoidance, conditioned taste aversion and appetitive conditioned approach response. Possible confounds in locomotor activity, un-conditioned fear/anxiety and motor coordination were assessed using the open field, elevated plus maze and accelerating rotarod, respectively. The present study adds to the argument that understanding adenosinergic regulation of behavior ought to take into account the intricate adenosinergic balance between telencephalon and structures beyond — in particular, the striatum.

2. Materials and methods

2.1. Animals

ADK^{TG} mice were created by breeding a loxP-flanked ADK transgene under the control of a human ubiquitin promoter into ADK knockout mice (Fedele et al., 2005; Yee et al., 2007). *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice were generated by breeding Emx1-Cre-TG3 mice, which express Cre-recombinase in neurons and astrocytes of the telencephalon (Iwasato et al., 2004), with *ADK*^{TG} mice (Li et al., 2008). *ADK*^{TG} and *ADK*^{TG}:*ADK*$^{Tel\text{-}def}$ mice produced as littermates and maintained on a C57BL/6 genetic background (Singer et al., 2012). Strain- and age-matched wild type (WT) mice served as controls. Littermates of the same sex were kept in groups of four to six in Type-III cages (Techniplast, Milan, Italy) housed in a temperature- and humidity-controlled (at 22 °C and 55% R.H.) animal vivarium under a reversed light–dark cycle (lights off from 0800 to 2000 h).

2.2. Behavioral testing

Behavioral testing began when the animals were approximately 12 weeks old, with all tests conducted in the dark phase of the light-dark cycle. Detailed information on animal cohorts, group sizes, and sequence of behavioral experiments is provided in Table 1. All experiments were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee adhering to NIH regulations and guidelines on the humane use of animals in research.

2.2.1. Open field locomotor activity

Four identical squared arenas (40×40 cm) as fully described by Hauser et al. (2005) were used. Locomotor activity was indexed by the distance traveled recorded in successive bins of 10 min over a test period of 1 h. Derivation of raw data was performed by the Ethovision tracking software (Noldus Technology, Wageningen, The Netherlands).

2.2.2. Elevated plus maze test of anxiety

The apparatus consisted of two exposed and two enclosed arms extending from a central square platform as described in detail elsewhere (Yee et al., 2004). The test lasted for 5 min during which the animal was allowed to freely explore the maze. Two anxiety-related measures were calculated: (i) percentage of time spent in the open arms = [time in open arms/time in open and enclosed arms×100%], and (ii) percentage of entries made into the open arms = [number of entries into open arms/number of entries into open and enclosed arms×100%]. In addition, the total distance traveled in the entire maze was recorded to index locomotor activity. All data were collected by the Ethovision tracking software (Noldus Technology, Wageningen, The Netherlands).

2.2.3. Accelerating rotarod

The Ugo-Basile Model 7650 (Comerio, VA, Italy) accelerating rotarod for mice was used to assess motor coordination and motor learning. Five subjects were tested concurrently and placed on the rotating drum at a baseline speed of 5 revolutions per minute (rpm). During the 5-min testing period, the speed was linearly increased to 40 rpm. The mice were given three trials with an inter-trial interval (ITI) of 24 h. The latency to fall from the rotating rod was recorded, with a maximum time of 300 s.

Table 1
Overview on experimental design, sequence of behavioral paradigms, number of cohorts, and group sizes in each test. [1] One female *ADK*^{TG} mouse fell from the plus maze and was therefore excluded from statistical analysis. [2] Animals from cohorts 1 and 2 were equally distributed between the two freezing experiments.[3] One female WT subject failed to learn the nose-poke response and was stopped from testing after pre-training.

Experiment	Duration (days)	Rest days before next test	Cohort	WT		*ADK*^{TG}		*ADK*^{TG}:*ADK*$^{Tel\text{-}def}$	
				Female	Male	Female	Male	Female	Male
Elevated plus maze	1	2	1	8	8	7[1]	7	4	7
Open field activity	1	2	1	8	8	8	7	4	7
Rotarod	1	–	1	8	8	8	7	4	7
Conditioned freezing									
CS freezing	3	–	1+2[2]	7	7	7	6	5	7
Context freezing	5	–	1+2	7	7	4	6	7	7
Appetitive conditioning	24	–	3	3[3]	3	5	3	5	3
Taste aversion	8	–	4	–	9	–	10	–	10
Active avoidance	5	–	5	3	3	4	4	4	4