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Working memory and the homeostatic control of brain adenosine by adenosine kinase

Philipp Singer^{1,2}, Stéphanie McGarrity^{1,3}, Hai-Ying Shen², Detlev Boison², and Benjamin K. Yee^{1,2}

¹Laboratory of Behavioural Neurobiology, Swiss Federal Institute of Technology, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland ²Robert Stone Dow Neurobiology Laboratories, Legacy Research Institute, 1225 NE 2nd Avenue, Portland, OR 97232, United States ³School of Psychology, University Park, The University of Nottingham, Nottingham, NG7 2RD, United Kingdom

Abstract

The neuromodulator adenosine maintains brain homeostasis and regulates complex behaviour via activation of inhibitory and excitatory adenosine receptors (ARs) in a brain region-specific manner. AR antagonists such as caffeine have been shown to ameliorate cognitive impairments in animal disease models but their effects on learning and memory in normal animals are equivocal. An alternative approach to reduce AR activation is to lower the extracellular tone of adenosine, which can be achieved by up-regulating adenosine kinase (ADK), the key enzyme of metabolic adenosine clearance. However, mice that globally over-express an *Adk* transgene ('Adk-tg' mice) were devoid of a caffeine-like pro-cognitive profile; they instead exhibited severe spatial memory deficits. This may be mechanistically linked to cortical/hippocampal *N*-methyl-D-aspartate receptor (NMDAR) hypofunction because the motor response to acute MK-801 was also potentiated in Adk-tg mice. Here, we evaluated the extent to which the behavioural phenotypes of Adk-tg mice might be modifiable by up-regulating adenosine levels in the cortex/hippocampus. To this end, we investigated mutant 'fb-Adk-def' mice in which ADK expression was specifically reduced in the telencephalon leading to a selective increase in cortical/hippocampal adenosine, while the rest of the brain remained as adenosine-deficient as in Adk-tg mice. The fb-Adk-def mice showed an even greater impairment in spatial working memory and a more pronounced motor response to NMDAR blockade than Adk-tg mice. These outcomes suggest that maintenance of cortical/hippocampal adenosine homeostasis is essential for effective spatial memory and deviation in either direction is detrimental with increased expression seemingly more disruptive than decreased expression.

Keywords

adenosine; learning; NMDA receptor; radial arm maze; glutamate; caffeine

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Correspondence to BKY at address² above, bbye@dowNeurobiology.org, +1 (503) 413 25 81.

Contributions:

PS and *SM* bred and genotyped the animals, performed all behavioural experiments, data collection and analysis; *HYS* provided solely access to immunostained materials in Figure 1; *DB* generated all transgenic animals employed in the study, and participated in manuscript preparation and interpretation; *BKY* & *PS* designed and planned the experiments, oversaw all statistical analysis and interpretation of all data including illustrations, and manuscript preparation.

The neuromodulator adenosine is not only critical for the maintenance of brain homeostasis but also the regulation of complex behaviours via its interaction with other neurotransmitters (Fuxe et al., 1998; Fredholm et al., 2005; Sebastião and Ribeiro, 2009). Rather than playing a central role in information processing, the influence of adenosine on cognitive processes is modulatory in nature, mediated primarily via its action on inhibitory A₁ receptors (A₁Rs) and excitatory A_{2A} receptors (A_{2A}Rs) expressed in neurons. Evidence for a critical involvement of adenosine in learning and memory is supported by behavioural data derived from animals as well as humans (for a review, see Boison et al., 2011). In particular, adenosine receptor (AR) antagonists are effective in ameliorating memory deficits in several animal models of degenerative diseases (e.g., Takahashi et al., 2008; Cunha and Agostinho, 2010). However, a clear consensus is lacking regarding whether similar pharmacological manipulations may yield pro-cognitive effects in normal unperturbed subjects (see Table 3 of Boison et al., 2011). Amongst these, the mixed A₁R/A_{2A}R antagonist, caffeine, has been more consistently reported to enhance performance on memory tests in normal animals, and it is commonly believed that such pro-cognitive effects stem primarily from an increase in arousal mediated via blockade of A_{2A}R signals in the nucleus accumbens (Fredholm et al., 1999; Nehlig, 2010). In agreement, selective genetic disruption of A_{2A}R in striatal neurons is sufficient to enhance working memory and to facilitate reversal learning (Wei et al., 2011).

As an alternative to chronic receptor blockade, we have been targeting adenosine kinase (ADK) – an astrocyte-based enzyme that catalyses the phosphorylation of adenosine, as a means to modify brain adenosinergic signalling (Boison, 2006; Etherington et al., 2009). Up-regulating ADK facilitates the clearance of intracellular adenosine by driving adenosine influx into astrocytes via bi-directional nucleoside transporters, and thus reduces extracellular adenosine levels (Boison et al., 2010). This has been achieved by replacing the endogenous *Adk* gene with an ubiquitin-driven loxP-flanked transgene [*Adk^{tm1}UbiAdk*], henceforth ‘Adk-tg’], which led to a 2.2-fold elevation in ADK activity and brain-wide reduction in adenosinergic tone (Fedele et al., 2005). Adk-tg mice were associated with severe performance deficits in the Morris water maze tests of spatial memory and Pavlovian conditioned freezing (Yee et al., 2007). The outcomes are therefore opposite to the pro-cognitive effects associated with AR blockade by caffeine (Yonkov, 1984; Cestari and Castellano, 1996; Kopf et al., 1999; Prediger and Takahashi, 2005; Prediger et al., 2005; Costa et al., 2008; Capek and Guenther, 2009, but also see Zimmerberg et al., 1991; Kant, 1993) and selective genetic inactivation of striatal A_{2A}Rs (Wei et al., 2011). Instead, they lend support to the hypothesis that physiological adenosine concentration is necessary for the homeostatic maintenance of neural network stability, including glutamatergic and dopaminergic networks, such that related behavioural outputs would be severely disturbed by the inhibition of adenosinergic neuromodulation (Yee et al., 2007; Boison et al., 2011).

The opposite outcomes between global ADK over-expression (Yee et al., 2007) and striatum-specific A_{2A}R disruption (Wei et al., 2011) regarding working memory performance suggest that the reduction in extra-striatal adenosinergic tone in the cortex and hippocampus might contribute to the behavioural deficits seen in Adk-tg mice. Within the hippocampus, adenosine modulates the function of ionotropic *N*-methyl-D-aspartate receptor (NMDARs) and metabotropic glutamate receptor 5 (mGluR5) via A₁R and A_{2A}R, respectively (Sebastião and Ribeiro, 2009). A substantial reduction of adenosinergic tone in the hippocampus may disrupt normal hippocampal function, including spatial learning. In parallel, the enhanced motor response to MK-801 (a non-competitive NMDAR blocker) seen in Adk-tg mice (Yee et al., 2007) is also suggestive of cortical/hippocampal NMDAR functional deficiency (Carlsson, 1993; Takahata and Moghaddam, 2003) – an effect not consistently associated with caffeine (see Table 1 of Boison et al., 2011) or reproducible by

striatal specific A_{2A}R disruption (Wei et al., unpublished data). Hence, the homeostatic modulation by cortical and hippocampal adenosine may be uniquely critical in this respect.

Here, we focused on the contribution of cortical/hippocampal adenosine reduction to the prominent behavioural phenotypes previously demonstrated in Adk-tg mice (Yee et al., 2007), namely, spatial memory and reaction to NMDAR blockade, and evaluated the extent to which they might be modifiable by cortical/hippocampal-specific interventions. To this end, we made use of the fact that the *Adk* transgene in the Adk-tg mice was floxed and therefore excisable by Cre recombinase. By restricting Cre expression to the telencephalon using the *Emx-1* promoter (Iwasato et al., 2004), we generated mice (denoted as 'fb-Adk-def') that lacked the *Adk* transgene specifically in the cortex and hippocampus while it remained overexpressed in the rest of the brain (i.e., similar to the original Adk-tg mice). Thus, fb-Adk-def mice were characterized by increased adenosine levels in cortex and decreased adenosine levels in striatum (Shen et al., 2011). With wild type (WT) mice providing the necessary control for gauging the direction of the phenotype relative to normal behaviour, the comparison between fb-Adk-def and Adk-tg mice allowed us to effectively compare opposite changes in cortical/hippocampal adenosine against the same background of adenosine deficiency outside the telencephalon. The regional molecular dissection achieved in the present study is therefore instructive in clarifying the direction and specificity of cognitive modulation maintained by cortical and hippocampal adenosinergic activity. This approach would be particularly relevant to models of disease, such as schizophrenia, that are hypothesized to be associated with a global adenosinergic hypofunction (e.g., Lara et al., 2006).

EXPERIMENTAL PROCEDURES

Adk-tg (*Adk*tm1^{-/-}:tg(UbiAdk)) 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). Fb-Adk-def (*Adk*tm1^{-/-}:tg(UbiAdk):*Emx1*-Cre-tg3) mice were generated by pairing *Emx1*-Cre-tg3 mice, which expressed Cre-recombinase in neurons and astrocytes of the telencephalon (Iwasato et al., 2004), with Adk-tg mice (Li et al., 2008). Adk-tg and fb-Adk-def mice were obtained as littermates. Control animals were derived from a matched WT C57BL/6 background maintained in parallel as previously described (Yee et al., 2007).

The regional alteration of ADK expression has been validated by immunohistochemistry as described before (Li et al., 2008; Shen et al., 2011). Here, we provide a new set of coronal sections taken at two different anterior-posterior levels obtained from the same animals (Figures 1A and 1B) with accompanying heat-map illustrations to demonstrate: (i) The brain-wide elevated expression of ADK in the Adk-tg mice was prominent in the striatum, extending to the ventral pallidum, and the entire cortical mantle and limbic cortices including hippocampus and amygdala, with milder increase seen in the thalamus and basal midbrain areas. (ii) The comparably prominent elevation of ADK expression seen in fb-Adk-def mice was restricted only to the striatal-pallidal structures, thalamus and mid-brain areas, but a clear deficiency relative to wild-type controls can be delineated throughout the entire cortical mantle extending from neocortical to allocortical structures including the hippocampus and the amygdala.

Animals

All mice were bred at the on-site specific-pathogen-free facility at the laboratory of Behavioural Neurobiology, ETH Zurich (Schwerzenbach, Switzerland), where all behavioural experiments described here took place. The animals were housed in a climatized vivarium (temperature ≈ 21°C, relative humidity ≈ 55%) under a reversed 12h/12h light-dark cycle (lights on at 8:00 pm). Behavioural testing commenced when the animals were 12

weeks old and took place during the dark phase of the light cycle. The group sizes in each of the three spatial learning tasks are summarized in Table 1. All procedures performed on the animals had been approved by the Zurich Cantonal Veterinary Office and were in compliance with the National Research Council's guide for the care and use of laboratory animals (2011).

Food deprivation

To motivate performance in the cheeseboard and radial arm maze (RAM) memory tasks, the animals were maintained on a 23 h food deprivation regime which was gradually introduced with a progressive reduction in daily feeding time: 12, 6, 4, 2, and 1 h/day. Afterwards, daily food rations (food pellets, Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) were calculated as a function of each animal's weight loss/gain from the previous day in order to maintain a stable weight of not less than 85% of the ad lib weight. Drinking water was available throughout. Animals maintained under food deprivation were individually caged whereas animals in all other tests were housed in groups of maximal 6 animals per cage of the same sex with ad libitum access to food and water.

Working memory in the radial arm maze (RAM)

The RAM had eight identical and equally spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side-length = 12 cm). Access to the arms from the central platform could be blocked by automated Plexiglas doors (12 cm wide, 16 cm high). Each arm had two Plexiglas side-walls decreasing circularly from a height of 16 cm at the arm's entrance to 1.5 cm. The rest of the arm was surrounded by a 5-mm high Plexiglas edge. At the far end of each arm, there was an automated pellet dispenser delivering food rewards (20 mg Noyes Food Pellets, Research Diets, NJ, USA) into a magazine tray. The experiment consisted of a habituation phase, followed by three specific working memory tests. The animals were given two days of rest between each working memory test.

•**Habituation (days 1–6)**—The animals were habituated to the maze for 5 min daily across 6 days. The animal was always released from the centre of the maze. On the first day, all doors were open and 16 pellets were randomly distributed over the 8 arms (2 per arm). On days 2–3, the animals were familiarized with the automated doors which randomly opened and closed during the 5-min exploration period. In addition, two pellets were now placed in each food magazine in order to encourage the animal to move to end of the arm. On days 4–6, four of the eight arms were randomly blocked by metal plates (12 wide, 16cm high) such that there was always an open arm adjacent to a blocked arm. The two possible configurations of 4 closed and 4 open arms were counterbalanced between genotypes. One pellet was placed in the food magazine of each open arm. A trial ended when all four pellets were consumed or a maximum of 5 min had elapsed.

•**4-arm test (days 7–22)**—The 4 open arms configurations of the maze were used here. At the start of a trial, the animal was placed in the centre of the maze with all doors closed. After 10 s, the four designated arms were opened and the animal could choose the first arm. An arm entry was scored when the animal passed the first quarter length into the arm (16 cm). Following this, all doors automatically closed confining the animal to the chosen arm. Once the animal approached the food magazine at the end of the arm the door of the chosen arm re-opened. By returning to the central platform the door closed again and the animal was confined there for 5 s. Afterwards, all doors re-opened and the animal could choose the next arm. A trial ended when all four rewards (one pellet per open arm) were consumed or 5 min had elapsed.

•**4-arms transfer test (days 23–26)**—Next, the configuration of the 4 open arms was reversed. Otherwise, the procedure remained unchanged.

•**8-arm test (days 27–34)**—By using all 8 arms the difficulty of the task was increased. The maximal time limit to consume the 8 food rewards was increased to 10 min.

The three working memory tasks were separately analysed. Working memory errors were defined as re-entries into previously visited arms and were calculated by the IMETRONIC (Pessac, France) computer program. Working memory performance was indexed by the “number of correct choices until the first error”, the “total number of errors”, and the “percentage error on total arm visits”. The different measures were separately analysed and yielded highly similar results. We opted to present in full details the results based on the “number of correct choices before the first error” because this measure (*i*) provides the clearest summary impressions of the experimental outcomes, and (*ii*) offers a unique advantage over the other two measures as it does not require any extrapolation when an animal fails to collect all rewards. In addition, the latency to complete the task was recorded as a measure of motivation.

Spatial reference memory in the cheeseboard maze

Spatial reference memory was assessed using the cheeseboard maze. The apparatus was identical to the one used by Lopez et al. (2010). In brief, it consisted of a circular wooden board, 1.1 m in diameter and 3 cm thick. On one side, there were 32 wells (3.1 cm in diameter and 1.3 cm deep) forming a radial pattern of 8×4 wells (5 cm apart) located at 20–45 cm from the centre of the maze. The reward consisted of two food pellets (25 mg, Noyes sucrose pellet) hidden inside the target well. To remove odour traces the entire maze was cleaned with 10% ethanol after each trial. On each trial, the latency and path-length to reach the reward were calculated using the Ethovision tracking software (Noldus Technology, Wageningen, the Netherlands).

•**Pre-training (days 1–5)**—Before testing commenced, the animals were habituated to the maze across five days. On each day, they were exposed to the undrilled side of the maze twice per day with an inter-trial interval (ITI) of 60 s. On each visit, the animal was placed in the centre of the maze and confined there by a semi-transparent plastic beaker (diameter: 15 cm, height: 25 cm). After 5 s, the beaker was removed and the animal allowed 120 s to explore the maze freely. To familiarize the animals with the food reward, 10 food pellets were randomly distributed on the maze on each trial.

•**Cued test (day 6)**—The animals were given two trials (ITI = 60 s) to locate the reward signalled by a visual cue (15 cm tall flag, 3×3 cm). On each trial, one random well was marked by the flag. A trial was completed when the animal consumed the reward or when 120 s had elapsed. Animals that failed to locate the reward within the allotted time were guided to it by the experimenter and a maximal latency of 120 s was scored.

•**Reference memory (days 7–18)**—The location of the reward was now fixed and no longer signalled by the visual cue. The eight wells located at a distance of 45 cm from the centre of the maze were used here. Assignment of the wells was counterbalanced between genotypes. There were two daily acquisition trials (ITI = 60 s) lasting a maximum of 120 s except trial 1 on day 7 and trial 2 on days 9 and 11 when a probe test was performed to assess short- and long-term memory retention. Each probe test lasted for 60 s during which no reward was available. The retention delay was 1 min in probe test 1 (day 7) because it began 1 min after trial 1's completion. Probe tests 2 and 3 were carried on day 9 and day 11, respectively, without any prior training on the same day. Hence, the retention interval was

effectively 24 h, and a normal training trial was conducted 1 min after the completion of probe tests 2 and 3. The sole purpose of the third and final probe test was to ascertain that the animals were relying on distal extra-maze cues to guide spatial navigation. This was achieved by rotating the cheeseboard maze by 180°.

To assess spatial search bias eight non-overlapping circular zones (diameter = 15 cm) were defined, each centring on the food well 45 cm from the maze centre at one of the eight possible directions. Search preference for the trained well was then indexed by the percentage time spent in the target zone of the total time spent in all eight zones [time in target zone/time in all eight zones × 100%].

Spatial familiarity judgment in the Y-maze

The ability to distinguish between novel and familiar space was assessed in the Y-maze as previously described (Pietropaolo et al., 2009). The test consisted of two phases separated by a variable time interval (delay). Each animal was assigned two arms (start arm and familiar arm) to which they were exposed during the sample phase. The remaining third arm constituted the novel arm to be used in the second phase (test phase). Allocation of arms (start, familiar, and novel) was counterbalanced within each experimental group. Access to the novel arm was blocked during the sample phase. To start a trial the animal was released from the end of the start arm facing the centre of the maze. After entering the familiar arm the animal was allowed to freely explore both the start and familiar arms for 5 min. In the test phase, the animal was returned to the maze for another 3-min exploration period with all arms accessible. Two delay intervals (30 min and 1 day) between sample and test phases were tested in two separate experiments using a between-subject design. During the delay, the animal was either kept in waiting cages in the testing room (30-min delay) or returned to the home cage (1-d delay). To avoid olfactory cues, the entire maze floor was covered with fresh saw-dust prior to each visit to the maze. Time spent in each arm was recorded for both sample and test phases by the Ethovision tracking software (Noldus Technology, Wageningen, The Netherlands).

Locomotor response to MK-801 in the open field

Locomotor activity was assessed in four identical open-field arenas measuring 40 × 40 × 35 cm as previously described (Yee et al., 2006). First, the animals were briefly acclimatized to the open field for 10 min. They were then removed and received a saline injection before being returned to the same open field arenas and observed for another 15 min. Next, they were once again removed and received either MK-801 (obtained from Sigma-Aldrich, Germany) at a dose of 0.15mg/kg via the intraperitoneal route at a volume of 5 ml/kg, or an equivalent volume of vehicle saline solution. The animals were immediately returned to the same open field and observed for 100 min. Locomotor activity was indexed by the distance travelled (in cm) and expressed in 5-min bins. Data were acquired by the Ethovision (Noldus, The Netherlands) tracking software. The three phases (i.e., naïve, saline, and drug) were analysed separately.

Data analysis

Parametric analysis of variance (ANOVA) was used to determine statistically significant differences. To further delineate the nature of significant outcomes, we conducted additional restricted analyses to subsets of the data included in the overall ANOVA, or pair-wise comparisons based on the associated error terms taken from the overall ANOVA. The results are presented as mean ± standard error (SE). Because the factor sex never significantly interacted with the effect of genotype, it was omitted to increase statistical power. The path-length measure obtained in the cheeseboard experiment was subjected to a square root transformation prior to statistical analysis in order to better conform to the

normality assumption of parametric ANOVA. All statistical analyses were carried out using PASW Statistics (version 18, SPSS Inc. Chicago IL, USA).

RESULTS

Spatial working memory is oppositely modulated by cortical/hippocampal and subcortical ADK/adenosine

Working memory is typically defined as the capacity to manipulate information held in short-term memory to perform complex tasks such as reasoning and problem-solving (Baddeley, 2003; Postle, 2006; Barch and Smith, 2008) and working memory deficiency represents a core cognitive symptom in schizophrenia (Park and Holzman, 1992; Lee and Park, 2005). Here, the animals were trained to enter each arm of the RAM to retrieve a food reward. To efficiently complete the task, they must avoid reentering a previously visited arm and this is expected to tax the on-going use of working memory buffer. The task started with only 4 arms and later included all 8 arms allowing us to assess the impact of increasing mnemonic load placed on the short-term memory buffer.

•4-arm test—Working memory performance was measured by number of correct choices until the first error. This is illustrated in blocks of two days in Figure 2A and was analysed by a 3×8 (genotype $\times$ block) ANOVA. Performance generally improved across blocks [$F(7,210) = 20.23$, $p < 0.001$] but was consistently reduced in the fb-Adk-def mice compared with the other two groups [$F(2,30) = 7.39$, $p < 0.005$]. This was further supported by post hoc pair-wise comparisons showing that the fb-Adk-def mice made significantly fewer correct choices until the first error than the other two groups (all p 's < 0.01). By contrast, the Adk-tg and WT mice did not significantly differ from each other ($p = 0.25$). Parallel analysis of the latency (in seconds) to consume the rewards failed to generate a significant genotype effect ($F < 1$) suggesting that the motivation was not affected by the mutations. The following mean latencies ($\pm$ SE) were obtained: WT = 4.18 ± 0.17 min, Adk-tg = 4.08 ± 0.21 min, fb-Adk-def = 4.45 ± 0.20 min.

•4-arm transfer test—Switching the configuration of the 4 open/4 closed arms had limited effect on performance irrespective of genotype (Figure 2A). All groups quickly adapted to this change in the experimental setting. As before, the fb-Adk-def mice consistently performed worse than the WT and Adk-tg mice yielding a significant main effect of genotype [$F(2,30) = 3.34$, $p < 0.05$] in a 3×2 (genotype $\times$ blocks) ANOVA. Post hoc pair-wise comparisons showed that the fb-Adk-def mice performed significantly worse than the other two groups (all p 's < 0.05). Similar analysis of the latency measure failed to yield a significant group difference ($F < 0.5$): WT = 3.81 ± 0.38 min, Adk-tg = 3.52 ± 0.47 min, fb-Adk-def = 4.01 ± 0.45 min.

•8-arm transfer test—Next, the difficulty of the task was increased by opening all eight arms. Performance generally increased across blocks but was worse in both mutant lines compared with the WT mice (Figure 2B). In particular, the fb-Adk-def mice showed a more severe deficit than the Adk-tg mice. Consistent with our interpretation, a 3×5 (genotype $\times$ blocks) ANOVA gave rise to a significant main effect genotype [$F(2,30) = 13.78$, $p < 0.001$] and blocks [$F(4,120) = 3.93$, $p < 0.01$]. Post hoc pair-wise comparisons revealed that both mutants groups made the first error earlier than the WT mice (all p 's < 0.05). The difference between the two mutant groups just approached statistical significance ($p = 0.05$). The three groups were again highly comparable in terms of latency to complete the task ($F < 0.5$): WT = 490 ± 21 s, Adk-tg = 499 ± 26 s, fb-Adk-def = 500 ± 25 s.

Acquisition and recall of spatial reference memory is not affected by changing in the ambient tone of adenosine

Reference memory was evaluated in the cheeseboard maze where the animals were trained to locate a food reward that remained in the same, fixed location on every trial. This task tests the ability to form associations between a spatial location and a certain outcome (e.g. a food reward)

•**Visible cue test**—To ascertain that the animals were able to perform the basic task, the location of the reward was first signalled by a local visible cue. The three groups equally performed on this task and quickly learned to locate the signalled reward as indicated by a reduction in the latency from trial 1 to trial 2 (Figure 3A). This was supported by a 3×2 (genotype $\times$ trials) ANOVA yielding only a significant main effect of trials [$F(1,31)=22.88$, $p<0.001$]. Running speed (m/s) significantly differed between genotypes [$F(1,31)=5.13$, $p<0.05$] mainly due to a lower speed in the fb-Adk-def mice (Table 2). Post hoc pair-wise comparisons revealed that the speed was significantly lower in fb-Adk-def than WT mice ($p < 0.05$) whereas the difference between the mutants groups was not statistically significant albeit there was a trend in the same direction ($p=0.09$).

•**Reference memory**—Across the next twelve days, the location of the food reward remained fixed and was no longer signalled by a visible cue requiring the use spatial cues to guide behaviour. Successful acquisition of spatial reference memory was reflected by a gradual reduction in the latency across days, and the rate of acquisition was comparable between groups (Figure 3B). Consequently, there was only a significant main effect of days [$F(8,16)=33.84$, $p<0.001$] in a 3×9 (genotype $\times$ days) ANOVA. At this stage of the task, running speed was constant across days and did not significantly differ between genotypes (Table 2). To assess memory retention, search bias for the trained target well was examined in a series of probe tests showing that spatial search accuracy was highly comparable between groups. Irrespective of retention interval (1 min or 24 h) or rotation of the maze (0 or 180°) the animals exhibited a clear preference to search in the zone centring on the target well (Figure 4). The three probe tests were analysed independently by separate 3×8 (genotype $\times$ zones) ANOVAs of the percentage of time spent in each zone. Consistent with our impression, the main effect of zone always attained statistical significance (probe 1: $F(7,217)=37.05$, probe 2: $F(7,217)=41.65$, probe 3 $F(7,217)=56.78$, all p 's <0.001). Running speed was higher in the mutant mice, especially the fb-Adk-def mice in comparison with WT controls (Table 2). A one-way ANOVA yielded a significant genotype effect [$F(2,31)=9.36$, $p=0.001$], and post hoc comparison confirmed that both mutants were moving faster than the WT mice [p 's <0.05].

Spatial familiarity judgment was indifferent to changing extracellular levels of adenosine

Spatial familiarity judgment was assessed by a novelty preference test in the Y-maze involving a choice between a previously visited (familiar) and a novel spatial location. A clear delay-dependent preference for the novel over the familiar arm was apparent regardless of genotype (Figure 5) giving rise to a significant main effect of arms $F(1,78)=56.37$, $p < 0.001$] and a delays $\times$ arms [$F(1,78)=4.50$, $p < 0.05$] interaction in a $3 \times 2 \times 2$ (genotype $\times$ delays $\times$ arms) ANOVA of the time spent in each of the two arms. Exploration of the familiar arm during the sample phase was comparable between groups in the two experiments (30-s vs. 24-h delays). WT, Adk-tg and fb-Adk-def mice spent on average 140 ± 6 s, 133 ± 6 s and 144 ± 6 s, respectively, exploring the arm that later served as the familiarized arm in the 30-min delay test condition. These figures are also comparable with those in the 24-h delay experiment: WT= 137 ± 6 s, Adk-tg= 138 ± 6 s, fb-Adk-def= 141 ± 6 s.

Enhanced response to MK-801 following global adenosine deficiency is exacerbated by telencephalon-specific up-regulation of adenosine

In the pre-injection phase, activity was similarly enhanced in both mutant lines relative to WT controls. These impressions were confirmed by post-hoc pair-wise comparisons [p 's < 0.005] following emergence of a significant genotype effect [$F(2,26)=11.12$, $p < 0.001$] (Figure 6).

In the saline phase, activity of Adk-tg and fb-Adk-def mice remained elevated relative to control as before, and showed a gradual increase across bins with WT controls remaining stably inactive. This gave rise to a significant genotype effect [$F(2,26)=22.67$, $p < 0.001$] and its interaction with bins [$F(4,52)=3.67$, $p = 0.01$]. Post hoc comparison confirmed that both mutants groups were significantly more active than controls (p 's < 0.005).

Next, MK-801 led to sustained increase in locomotor activity from the sixth bin into the drug phase of the experiment. With initial level of activity now becoming comparable between genotypes, it was apparent that the drug-induced hyperactivity was potentiated in both mutant lines, and this effect was clearly stronger in the fb-Adk-def mice than Adk-tg mice. This was confirmed by the highly significant genotype effect [$F(2,26)=18.15$, $p < 0.001$], and post-hoc comparisons which indicated that all three genotypes significantly differed from one another [all p 's < 0.05]. Thus although the two mutant lines were largely similar in the pre-injection and saline phase, they were clearly distinguishable in the drug phase by the magnitude of their hyperactive response to MK-801.

DISCUSSION

Here, global adenosine deficiency following brain-wide up-regulation of ADK led to significant impairment in spatial working memory in the Adk-tg mice without any apparent impact on spatial reference memory in or spatial familiarity judgment. The latter outcomes exclude the possibility that the working memory deficiency stemmed from non-specific motor deficits, impairment in the sensory perception of spatial cues, or lack of motivation to seek food reward. By comparison, region-specific up-regulation of adenosine in the cortex and hippocampus within the Adk-tg background – as a result of telencephalon-specific disruption of the ADK transgene, exacerbated rather than ameliorated the working memory deficit associated with brain-wide over-expression of ADK in Adk-tg mice. Likewise, the hyper-responsiveness to MK-801 seen in Adk-tg mice was exacerbated by the local increase in extracellular adenosine introduced specifically to the telencephalon. Thus, the magnitude of the working memory deficit and the sensitivity to NMDAR blockade seemed to go hand in hand. Although the present study cannot ascertain the existence of a causal link between NMDAR hypofunction and working memory deficit in our mutant mice, this speculation certainly warrants serious consideration. It may be tested by assessing whether drugs that enhance NMDAR function, such as glycine transporter 1 inhibitor therapy (Singer et al., 2009), would ameliorate the memory deficits.

Although their phenotypic profile was similar, the impression that Adk-tg and fb-Adk-def mice were clearly distinguishable by the severity of their phenotypic expression excluded the possibility that their common adenosine over-expression outside the telencephalon (e.g., the striatum) could be solely responsible for the phenotypes demonstrated here. Such a view would also be hard to reconcile with the consensus that blockade of striatal adenosine receptors (ARs), in particular A_{2A} Rs, has been associated with performance enhancement in working memory tests (Takahashi et al., 2008; Wei et al., 2011). Indeed, any difference in phenotypic expression between the two mutant lines must be attributable to the local *Adk* deletion introduced to the telencephalon that sets the two apart from each other. As will be

discussed later, this is not to deny that subcortical/striatal adenosine normally may also modulate cognitive performance.

Specificity of working memory impairment

Spatial working memory was assessed in the RAM test in which the animals had to keep track of the arms already visited so as to avoid them in the subsequent choice on a given daily trial. Reliable avoidance of re-entry errors depends on an effective short-term memory buffer that enables the distinction between visited (familiar) and un-visited (novel) arms within a given trial. The animals readily acquired the task when they were trained initially on the 4-arm version of the test which seemed to pose limited demand on the working memory buffer: Adk-tg mice performed on a par with WT controls with fb-Adk-def mice trailing behind yet showing some evidence of improvement over time (Figure 2A). Next, the transfer test results showed that all mice had mastered the rules of the test. The critical finding here emerged when all eight arms were included in the test, thus doubling the demand on the working memory buffer in terms of the number of arms needed to be held on-line to support errorless performance. Now, fb-Adk-def and Adk-tg mice were both clearly impaired, with the deficit in fb-Adk-def mice being significantly more severe (Figure 2A), thus confirming the initial impression obtained in the 4-arm version of the test. Unlike GluR1 knock-out mice whose working memory deficiency has been linked to impaired short-term familiarity judgement as demonstrated by their Y-maze performance (Reisel et al., 2002; Sanderson et al., 2007, 2010), both of our mutants here performed like WT mice in the Y-maze test of spatial familiarity judgement (Figure 5). The fact that sufficient demand on storage was necessary to solicit a clear deficit in the mutant mice suggests that the impairment may instead reflect a deficiency in the capacity of the memory buffer. This is the first suggestion of a specific link between cortical/hippocampal adenosine and short-term memory storage capacity. To further dissect the relative contributions of ADK/adenosinergic modulation within discrete telencephalonic structures to spatial working memory, for instance hippocampus or prefrontal cortex, region-specific manipulation of ADK/adenosine would be necessary. This may be achieved by local application of adeno-associated virus-based vectors engineered to overexpress or knockdown *Adk* (Shen et al., 2011) or intracerebral transplantation of adenosine-releasing cells into distinct brain regions (Boison, 2007).

Given that spatial recognition memory in the Y-maze was preserved in Adk-tg and fb-Adk-def mice it is not surprising that no evidence of a reference memory deficit emerged in the cheeseboard task across all stages of the test (Figures 2 and 3). Performance in this test depends not on flexible short-term memory but on long-term memory of a constant association between a single location and the availability of a food reward. The normal performance by the mutants indicated that neither long-term memory nor spatial navigation was affected, strengthening the specificity of the working memory phenotype. However, the absence of an effect in the cheeseboard here is at odd with the previous water maze experiment reporting a severe reference memory deficit in Adk-tg mice (Yee et al., 2007). This discrepancy likely stemmed from experimental confounds in the previous study: Yee et al. (2007) evaluated reference memory in animals that had been subjected to working memory test previously in the same water maze. Given that the Adk-tg mice already performed poorly in the working memory test, their motivation to escape might have been compromised, not to mention the possibility of negative transfer between the two versions of tests due to incompatible strategies (win-shift vs. win-stay between days). In contrast, the current working memory and reference memory tests were conducted in naïve subjects, and therefore free from such possible transfer effects that could have confounded our results. There is however other confounding that still warrants consideration. For instance, one might not rule out the possibility that the divergent effects in the two reference memory

tasks might stem from a difference in the arousal nature of the water maze tests due to its aversive nature (Lyon et al., 2011), or motivational requirements (negative vs. positive reinforcement), or perhaps task difficulty. Nonetheless, the present study has provided clear confirmation of the robustness of Yee et al.'s (2007) initial report of working memory deficiency in the water maze.

Imbalance of adenosine receptor signalling due to abnormal ADK expression

The neuromodulatory effects of adenosine are mainly mediated via A₁R and A_{2A}R. Although manipulating ADK should not discriminate between the two receptor subtypes, their relative recruitment is dependent on extracellular adenosine concentration. Hence, to fully appreciate the physiological mechanisms whereby changes in ADK expression might bring about the observed behavioural effects, it is necessary to consider A₁R and A_{2A}R mechanisms as well as their interaction.

Under physiological levels of extracellular adenosine, A₁R activity in cortex/hippocampus prevails providing a tonic inhibition over glutamate release and excitatory neurotransmission. Upon rapid neuronal firing, the accompanying surge in ATP-derived extracellular adenosine would recruit local postsynaptic A_{2A}Rs, which inhibit A₁Rs and thereby enhance NMDAR function and facilitate long-term potentiation (LTP) at the stimulated synapses. Together, A₁R and A_{2A}R provide a gain control mechanism to reduce noise and enhance signal detection by increasing salience between activated and non-activated synapses (Fredholm et al., 2005; Cunha, 2008; Rebola et al., 2008). This intricate balance would be disrupted when ambient adenosine concentration is altered in either direction. The indiscriminate reduction in A₁R and A_{2A}R activation resulting from lower extracellular adenosine in our Adk-tg mice (Fedele et al., 2005) may be approximated by the systemic action of the mixed A₁R/A_{2A}R antagonist, caffeine, which depresses hippocampal LTP primarily via A_{2A}R antagonism (Constenla et al., 2010). Specifically, blockade of CA3 A_{2A}R is sufficient to disrupt NMDAR-dependent LTP at dentate-CA3 synapses (Rebola et al., 2008), the integrity of which is essential for spatial working memory (Nakazawa et al., 2002, 2003). Hence, impaired working memory in Adk-tg mice is explicable in terms of under-activation of cortical/hippocampal A_{2A}R rather than that of A₁R, since blockade of the latter enhances LTP (de Mendonca and Ribeiro, 2001). The normal spatial working memory performance following A₁R deletion (Giménez-Llort et al., 2007) also indirectly favours an A_{2A}R-dependent mechanism being responsible for the deficits observed in our Adk-tg mice.

By contrast, telencephalon-specific ADK deletion in our fb-Adk-def mice should lead to cortical/hippocampal over-activity of A₁R and A_{2A}R. Stimulation of hippocampal A₁R but not A_{2A}R by adenosine suppresses LTP formation *in vitro* (Arai et al., 1990; de Mendonca and Ribeiro, 2001), which is consistent with the general consensus that LTP is attenuated by A₁R agonists but facilitated by A₁R antagonists (de Mendonca and Ribeiro, 2001). Of relevance here is that intra-hippocampal infusion of A₁R- but not A_{2A}R-selective agonists impaired working memory (Ohno and Watanabe, 1996). Thus, the behavioural phenotypes of fb-Adk-def mice resemble the effects of cortical/hippocampal A₁R rather than A_{2A}R over-activation. This complements the A_{2A}R-dependent mechanisms proposed above in the context of Adk-tg mice. Thus, normalization of A₁R activity alone in fb-Adk-def mice might be sufficient to correct their behavioural dysfunction. We therefore venture to hypothesize that the working memory phenotypes of Adk-tg and fb-Adk-def mice might be attributed to A_{2A}R under-activity and A₁R overactivity in the cortex/hippocampus, respectively. In addition, the similarity between their phenotypic profiles might be linked to a common impairment of cortical/hippocampal NMDAR-dependent neuroplasticity. Our empirical evidence further suggests that A₁R over-activity is more detrimental to working memory than A_{2A}R under-activity. The former is consistent with the pro-cognitive profile

A₁R antagonists (Stone et al., 1995; Ribeiro et al., 2003; Ribeiro and Sebastião, 2010), and we may add to this A_{2A}R agonism as a possible complementary pro-cognitive strategy.

Contributions of subcortical adenosine

In addition to its modulatory effect of cortical/hippocampal synaptic plasticity, adenosine regulates arousal through its influence on subcortical brain activity, which may indirectly modify overt performance (Fredholm et al., 1999; Nehlig 2010). Indeed, the dose-response function of caffeine in healthy humans follows an inverted U-shaped relationship (Nehlig, 2010) in accordance to the Yerkes-Dodson's law (1908) between arousal and performance. Caffeine reportedly improves performance on simple tasks with a low demand on working memory but yields no effect or even impairs performance on high-demand tasks (for reviews see, Fredholm et al., 1999; Nehlig, 2010). Furthermore, the arousal effect might be primarily related to the acute action of caffeine given that tolerance rapidly develops to chronic caffeine exposure (Holtzman et al., 1991; Johansson et al., 1997; Svenningsson et al., 1999). A variety of compensatory changes such as alterations in AR expression may underlie caffeine tolerance (Guillet and Kellogg, 1991; Johansson et al., 1997; Svenningsson et al., 1999) and perhaps explain the different, or even opposite, behavioural effects of acute vs. chronic caffeine treatment (Nehlig et al., 1992; Corodimas et al., 2000; for a review see Jacobson et al., 1996). Such compensatory mechanisms are also expected in genetically modified animals, which may account for the inconsistent results in the literature between mouse mutant and acute pharmacological studies (Boison et al., 2011). Despite such discrepancies, the current consensus is that caffeine stimulates arousal primarily by blocking A_{2A}R in the striatum (Chen et al., 2010) and recent data derived from A_{2A}R-selective knockouts have shown that inactivation of striatal A_{2A}Rs is sufficient to enhance learning performance including spatial working memory (Wei et al., 2011). It is therefore unlikely that the working memory impairment seen in Adk-tg mice here stems primarily from a hypofunction of subcortical/striatal A_{2A}Rs and thus further supports a cortical/hippocampal mechanism.

Developmental changes and compensations

Unlike pharmacological treatment in adulthood, Adk-tg and fb-Adk-def mice are also susceptible to early life developmental changes. The loss of the endogenous Adk gene in Adk-tg and fb-Adk-def mice was constitutive, and the ubiquitin-driven global expression of the Adk-transgene is expected to occur as early as embryonic day 1 (E1). It follows that potential developmental changes occurring until E10 should be similar in the two mutant lines. The telencephalon-specific stoppage of ubiquitin-driven expression of the Adk-transgene controlled by EMX-1 in the fb-Adk-def mice came later at E10–E11 (Iwasato et al., 2004). Thus, possible developmental divergence between the two lines might be expected post-E10. It is therefore worth noting that a critical developmental event involves a shift from ADK expression in both neurons and astrocytes to solely astrocytic expression by postnatal day 21 (Studer et al., 2006), suggesting distinct functions between the immature and mature brains: While astrocytic ADK regulates the extracellular adenosine concentration in the adult, expression of ADK in immature neurons regulates development and plasticity (Studer et al., 2006). Hence, interpretation of the phenotypic differences observed in our adult mutants needs to consider such caveats common to genetic mouse models. Models based on inducible region- and cell-type-specific ADK deletion should resolve such concerns.

CONCLUSIONS

The present data suggest that homeostatic balance of the cortical/hippocampal adenosinergic tone is necessary for normal working memory function and any deviation appears to impair

performance – either as a consequence of A_{2A}R under-activation when adenosine levels are reduced or due to enhanced A₁R-mediated inhibition when adenosine levels are elevated. Either of these represents also an imbalance between cortical/hippocampal and striatal adenosinergic regulation due to the differential expression of the two receptor subtypes in the striatum and cortex/hippocampus. Indeed, this particular regional imbalance may explain the stronger memory impairment seen in fb-Adk-def mice compared with Adk-tg mice. It therefore follows that (i) rebalancing or stabilizing cortical/hippocampal adenosinergic tone might be beneficial in correcting working memory deficiency in a number of cognitive disorders where adenosine homeostasis may be disturbed (for a review, see Boison, 2008), and (ii) boosting A_{2A}R-mediated signalling in the striatum may independently confer additional pro-cognitive benefits possibly via an independent mechanism. More specific manipulations in terms of brain regions (cortical vs. striatal) and AR subtypes (A₁R vs. A_{2A}R) are needed to verify our novel hypothesis regarding adenosine's multiple regulatory roles in learning and memory.

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ABBREVIATIONS

A₁R	adenosine A ₁ receptor
A_{2A}R	adenosine A _{2A} receptor
ADK	adenosine kinase
Adk-tg	<i>Adktm1^{-/-}; Tg(UbiAdk)</i>
ANOVA	analysis of variance
AR	adenosine receptor
fb-Adk-tg	<i>Adktm1^{-/-};tg(UbiAdk);Emx1-Cre-tg3</i>
ITI	inter-trial interval
LTP	long-term potentiation
mGluR5	metabotropic glutamate receptor 5
NMDAR	<i>N</i> -methyl-D-aspartate receptor
RAM	radial arm maze
SE	standard error
WT	wild type

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HIGHLIGHTS

- Global reduction in brain adenosine impairs specifically working memory
- Elevation of cortical adenosine yielded the same but more severe phenotype
- Under- and over-activation of A₁ and A_{2A} receptors, respectively, appear crucial
- Normal working memory requires cortical/hippocampal adenosine homeostasis

Figure 1.

Photomicrographs of ADK immunoreactivity in coronal brain sections of adult mice, processed for immunoperoxidase staining under identical conditions, and corresponding heat-map of stain intensity. Immunoreactivity (ir) was visualized by DAB reaction product (top row in **A** and **B**) as fully described by Shen et al (2011). Heat maps (bottom row in **A** and **B**) illustrating the differential intensity of ADK-ir of the corresponding photomicrographs across genotypes were generated by the application of the pre-set gradient map in Photoshop CS4 to the entire set of digitized photomicrographs. Sections illustrated in **A** approximately correspond to the anterior-posterior (A-P) level of +0.74 mm relative to Bregma, while sections illustrated in **B** approximately correspond to A-P -1.64 mm relative to Bregma (estimated according to the Mouse Brain Library C57BL6 brain atlas (http://www.mbl.org/atlas170/atlas170_start.html) to highlight the alterations in the striatal complex and cortex/hippocampus, respectively. The three genotypes are arranged in separate columns across here, with Adk-tg mouse on the left and fb-Adk-def mouse on the right, to facilitate comparison with wild-type (WT) control positioned in the middle. It can be readily discerned that Adk-ir was elevated in the entire brain of Adk-tg mice. Similarly levels of elevation was observed in the striatal-pallidal structures, thalamic and basal midbrain regions of fb-Adk-def mice, which simultaneously displayed a notable deficiency of Adk-ir in the cortical mantle, hippocampus and amygdala relative to the WT control.

Figure 2.

Working memory performance in the RAM is indexed by the number of correct choice until the first error. Thus, the higher the score the better is the performance. **(A)** Performance was consistently impaired in the fb-Adk-def mice in comparison to the WT and Adk-tg mice in the 4-arm test as well as the 4-arm transfer test. **(B)** Both mutant groups exhibited a working memory deficit relative to the WT mice when all eight arms of the maze were used.* denotes a significant group difference ($p < 0.005$) based on post hoc pair-wise comparisons following the emergence of a significant genotype effect in the overall ANOVA. Values are mean $\pm$ SE.

Figure 3.

The two phases of the cheeseboard experiment. **(A)** All groups equally learned to follow the visible cue to obtain a reward across the two trials. **(B)** Successful acquisition of reference memory is indexed by a gradual improvement across days. The rate of acquisition was comparable between groups. **(C)** spatial search accuracy in the probe tests as indexed by the percentage of time spent in each of the eight circular zones centring on the food wells (Tg = target well, L1–L3 = three wells on the left of Tg, R1–R3 = three wells on the right of Tg, Op: well opposite of Tg). Irrespective of genotype, clear search bias for the target zone was detected across the three probe tests. Values are mean $\pm$ SE

Figure 4.

Spatial search accuracy in the probe tests as indexed by the percentage of time spent in each of the eight circular zones centring on the food wells (Tg = target zones, L₁–L₃ = three zones on the left of Tg, R₁–R₃ = three zones on the right of Tg, Op: the zone opposite to Tg). Irrespective of genotype, clear search bias for the target zone was detected across the three probe tests (A to C). Values are mean ± SE.

Figure 5.

Spatial recognition memory as indicated by an explorative preference towards the novel (N) over the familiar (F) arm. * denotes a significant arm effect ($p < 0.05$) according to the respective ANOVA. Values refer to mean $\pm$ SE.

Figure 6.

Locomotor response to systemic MK-801. Locomotor activity as indexed by the distance travelled per 5-min bin is shown for the pre-injection phase (bins 1 to 2), the saline phase (bins 3 to 5) and the drug phase (bins 6 to 26). The bar plot on the right illustrates the mean distance travelled per bin during the drug phase. Both mutants showed increased activity relative to controls in all three phases. In the naïve and the saline phases, # denotes that controls significantly differed from Adk-tg and fb-Adk-def mice based on post hoc comparisons ($p < 0.05$). The two mutants became distinguishable under the influence of systemic MK-801 (0.15 mg/kg). The drug-induced motor response was potentiated in Adk-tg mice, and this was exacerbated even further in fb-Adk-def mice. This is illustrated in the bar plot on the right (* indicates $p < 0.05$ based on post hoc comparison following the emergence of a significant genotype effect). All values refer to mean $\pm$ SE.

Table 1

Number of subjects accepted in the final analyses of each experiment. The three experiments were performed in separate cohorts of animals.

Test Paradigm	Behavioural readouts	WT		Adk- <i>tg</i>		Fb-Adk-def	
		♀	♂	♀	♂	♀	♂
Radial arm maze	Working memory	8	8	7	7	5	7
Cheeseboard	Reference memory	17	15	14	15	11	15
Y-maze	Recognition memory	-	14	-	9	-	10
Open field	Response to MK-801	5	6	6	5	5	5

Table 2

Running speed in m/s in the different phases of cheeseboard experiment. Values refer to mean $\pm$ SE.

Genotype	Cued test	Reference memory	
		Acquisition	Probe test
WT (n=13)	5.86 $\pm$ 0.52	8.65 $\pm$ 0.56	8.54 $\pm$ 0.78
Adk-tg (n=12)	5.22 $\pm$ 0.62	9.64 $\pm$ 0.68	11.53 $\pm$ 0.94
Fb-Adk-def (n=9)	3.78 $\pm$ 0.54	10.45 $\pm$ 0.59	12.30 $\pm$ 0.81