Cognitive impairments associated with alterations in synaptic proteins induced by the genetic loss of adenosine A_{2A} receptors in mice

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Abbreviations: Attention deficit/hyperactivity disorder (ADHD), Bromodeoxyuridine (BrdU), Central nervous system (CNS), Conditioned stimulus (CS), Cornu ammonis 1 (CA1), Cornu ammonis 3 (CA3), Dentate gyrus (DG), Inter-trial interval (ITI), Knock-out (KO), Phosphate buffer (PB), Prefrontal cortex (PFC), Tris-buffered saline (TBS), Unconditioned stimulus (US), Wild-type (WT).

Abstract

The study of psychiatric disorders usually focuses on emotional symptoms assessment. However, cognitive deficiencies frequently constitute the core symptoms, are often poorly controlled and handicap individual's quality of life. Adenosine receptors, through the control of both dopamine and glutamate systems, have been implicated in the pathophysiology of several psychiatric disorders such as schizophrenia and attention deficit/hyperactivity disorder. Indeed, clinical data indicate that poorly responsive schizophrenia patients treated with adenosine adjuvants show improved treatment outcomes. The A_{2A} adenosine receptor subtype (A_{2A}R) is highly expressed in brain areas controlling cognition and motivational responses including the striatum, hippocampus and cerebral cortex. Accordingly, we study the role of A_{2A}R in the regulation of cognitive processes based on a complete cognitive behavioural analysis coupled with the assessment of neurogenesis and sub-synaptic protein expression in adult and middle-aged A_{2A}R constitutional knockout mice and wild-type littermates. Our results show overall cognitive impairments in A_{2A}R knockout mice associated with a decrease in new-born hippocampal neuron proliferation and concomitant changes in synaptic protein expression, in both the prefrontal cortex and the hippocampus. These results suggest a deficient adenosine signalling in cognitive processes, thus providing new opportunities for the therapeutic management of cognitive deficits associated with psychiatric disorders.

Running title: Adenosine A_{2A}R and cognitive impairments in mice

Keywords: Adenosine A_{2A}, cognition, aging, neurogenesis, synaptic function

Highlights:

1. Deletion of A_{2A}R induces overall cognitive deficits in adult and middle-aged mice.
2. Deficits in cognition are associated to decreases in neurogenesis.
3. A_{2A}R deletion induces changes in synaptic proteins in the PFC and hippocampus.

1. Introduction

Psychiatric, neurological and substance use disorders are the leading cause of the disease burden worldwide (Whiteford et al., 2015). However, while the study of psychiatric disorders focused mostly on the emotional symptoms assessment (Millan et al., 2012), in some cases (i.e. schizophrenia), cognitive alterations constitute the core symptoms of the disease (Fioravanti et al., 2012; Napal et al., 2012). Moreover, other psychiatric disorders such as major depression, bipolar disorder, attention deficit/hyperactivity disorder (ADHD) or autism spectrum disorder also exhibit a wide range of cognitive symptoms that are often disregarded (Millan et al., 2012). Such deficits include changes in working memory, executive functioning or emotional memory, which often handicap the individual's quality of life. Importantly, these cognitive alterations do not tend to improve with treatment (Hill et al., 2010), and defining the specific cognitive domains impaired in each disease may also be difficult. Furthermore, some of the aforementioned diseases, such as schizophrenia, progress with neurodegeneration, thus causing brain damage similar to that shown in Alzheimer's or Parkinson's disease (Lieberman, 1999; Perez-Neri et al., 2006). Hence, the study of the age-associated disease progression should be considered when assessing cognitive deficiencies.

Alterations in the adenosinergic system have been implicated in brain functions related to psychiatric disorders such as schizophrenia and ADHD (Boison et al., 2012; Cunha et al., 2008). Interestingly, clinical data indicate that poorly responsive schizophrenia patients treated with adenosine adjuvants show improved treatment outcomes (Akhondzadeh et al., 2000; 2005). Adenosine is an endogenous purine nucleoside acting at the central nervous system (CNS) exerting its biological effects through the activation of four G protein-coupled receptors, namely adenosine A₁, A_{2A}, A_{2B} and A₃ receptors (A₁R, A_{2A}R, A_{2B}R and A₃R, respectively) (Fredholm et al., 2001). A_{2A}Rs modulate neuronal activity by regulating glutamate and dopamine neurotransmission (Shen and Chen, 2009) and have been involved in the pathophysiology of psychiatric disorders, traumatic and ischemic brain injury, and neurodegenerative diseases (Cunha et al., 2008; Moscoso-Castro et al., 2016). Intriguingly, A_{2A}Rs are highly expressed in striatal neurons and, to a lesser extent, in

other types of neurons, including the hippocampus, cerebral cortex and glia (Svenningsson et al., 1997), and brain areas involved in the control of motivation, executive functioning or learning and memory (Burgess et al., 2002; Funahashi and Andreau, 2013).

In the present study we aimed to assess the role of A_{2A}R in the regulation of cognitive processes. To this end, we first performed a broad cognitive behavioural assessment of adult and middle-aged A_{2A}R constitutional knockout (KO) mice and wild-type (WT) littermates. Subsequently, we evaluated neurogenesis and expression of key synaptic proteins involved in learning and memory processes (Eastwood et al., 1995; Corradi et al., 2008; Schmitt et al., 2009). Therefore, the levels of extra-, pre- and postsynaptic enriched proteins (i.e. synaptophysin, SNAP25 and PSD95, respectively) in the hippocampus and prefrontal cortex (PFC) were analysed to evaluate age-associated cognitive decline by a decrease in new-born hippocampal neuron proliferation and changes in synaptic protein expression in A_{2A}R knockout mice.

2. Materials and Methods

2.1 Animals

45 adult and 50 middle-aged A_{2A}R KO male mice and 44 adult and 40 middle-aged A_{2A}R WT littermates with a CD1 background were used in our experiments. A_{2A}R KO mice were generated in the University of Barcelona animal facility with a CD1 background as previously described (Ledent et al., 1997). When the experiments commenced, the adult animals were 12 weeks old, while the aged animals were 12 months old. A different set of mice was used for studies in adult and middle-aged animals. The animals were housed in a temperature ($21^{\circ} \pm 1^{\circ} \text{C}$) and humidity-controlled room ($55\% \pm 10\%$), and subjected to a fixed 12-h light/dark cycle with the lights on between 8.00 am and 8.00 pm. Food and water were available *ad libitum* except when radial arm maze was performed, as described below. All animal care and experimental procedures were conducted according to the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEAA-PRBB).

2.2 Drugs

Ketamine hydrochloride (100 mg/kg; Imalgène 1000®, Merial, Toulouse, France) and xylazine hydrochloride (20 mg/kg; Sigma, Madrid, Spain) were mixed in ethanol and water (1:9). This anaesthetic mixture was injected in a volume of 0.2ml/10 g body weight i.p. and used for intracardiac perfusion.

2.3 Spontaneous alternation Y maze test

Mice were tested in the spontaneous alternation Y maze test in order to assess working memory (Lalonde, 2002) using a Y-shaped maze. The grey plastic maze had three identical arms (40 x 6 x 15 cm, L x W x H) separated by 120° angles and the mice were placed in the centre and allowed 8 minutes for free exploration. The number and sequences of arm visits were recorded manually by the observer. An alternation was defined on the basis of consecutive entry in the three different arms. The ratio of alternations was computed with the formula: number of alternations divided by total number of alternations minus 2, as previously reported (Arai et al., 2001).

2.4 Radial arm maze

The radial arm maze (Panlab s.l.u., Barcelona, Spain) was conducted as previously described with minor modifications (Ros-Simó et al., 2013). Briefly, the maze consisted of a central hub with eight arms placed in a high tripod, although in order to evaluate cognitive alterations in adult and aged mice, only six arms were used. The maze was surrounded by black curtains where three extra-maze black and white visual cues were placed. Food wells at the tip of each arm were used for the supply of chocolate pellets (Choco Krispis, Kellogg's). Animals were placed in the centre of the maze and were trained to find the food pellets situated in three randomly selected arms. Each acquisition session consisted of either two 5 min trials/day or until the mice found all the pellets for 19 consecutive days. Spatial memory was assessed with reference memory errors (i.e., entries into non-baited arms).

2.5 Electrical nociceptive threshold

Pain threshold was assessed in adult mice, as previously described (Gracia-Rubio et al., 2016; Martin et al., 2002;). Briefly, mice were allowed 5 min of habituation in the

dark compartment of the passive avoidance box prior to the application of a series of 10 inescapable shocks. The locomotor activity during habituation, number of jumps, and presence (1) or absence (0) of vocalizations was assessed. Electrical foot shocks were spaced at 30 s and delivered at an intensity of 0.5 mA for 3 s. The animals used for this procedure were not used for any other behavioural studies.

2.6 Passive avoidance test

The passive avoidance test was used to assess emotional learning and memory based on the natural preference of mice for dark environments, and the posterior association between an aversive stimulus (e.g. foot shock) and the preferred environmental context. The experiment was conducted as previously described (Saavedra et al., 2013) in a two-compartment device with varying luminosity (dimly lit compartment: 2–5 lux; and brightly lit compartment: 160 lux; dimensions, 19 x 19 x 27 cm) (Panlab s.l.u., Barcelona, Spain). On the acquisition day, animals were placed in the brightly lit compartment. After 30 s, the sliding door separating both compartments was opened to allow access to the dimly lit compartment and the latency to enter was registered for a maximum of 90 s. Animals were then given an inescapable foot shock (0.5 mA), for 3 s. The retention test was conducted 24 h after the acquisition session (with no shocks) and the latency time to enter the dark compartment was recorded with a cut-off of 300 s.

2.7 Active avoidance test

A two-way shuttle box apparatus (Panlab s.l.u., Barcelona, Spain) was used to assess two-way active avoidance learning in mice. The procedure was conducted as previously reported with minor modifications (Martin et al., 2002). Briefly, mice were trained to avoid an aversive unconditioned stimulus (US) - an electric shock (0.2 mA) applied to the grid floor following the activation of a light serving as a conditioned stimulus (CS). The shuttle box consisted of two compartments (19 x 19 x 27 cm) divided by a vertical wall with a central opening allowing for free movement between compartments. Each session began with a habituation period of 5 min followed by 50 trials, separated by an inter-trial interval (ITI) of 30 s. In each trial, the light serving as a CS was presented for 20 s in the compartment where the animal was situated. After 10 s, the foot shock was applied. Immediately after crossing to the other compartment, the CS and the foot shock were automatically turned off and the ITI

began. Animals could learn to avoid the shock either by crossing to the opposite compartment after the presentation of the CS or once the foot shock began. In the first case, it was scored as a conditioned response and in the second as an unconditioned response. When animals failed to cross to the opposite compartment, it was scored as a non-response. Two separate groups of animals underwent the active avoidance test during adulthood and at middle-age.

2.8 Bromodeoxyuridine administration and brain tissue sample preparation

To determine neural cell proliferation, animals were injected three times a day at 2-h intervals with bromodeoxyuridine (BrdU) (100 mg/kg, i.p.), as previously described, with minor modifications (Chen et al., 2012; Johansson et al., 2015). Animals were then sacrificed either 3 or 30 days after the final BrdU injection to determine neurogenesis proliferation and survival respectively.

2.9 Immunohistochemistry

In order to quantify neurogenesis we used rat anti-BrdU (1:500, Cat. Ab6326, Abcam, Cambridge, UK) and mouse anti-NeuN (1:500, clone A60; Cat. MAB377, Merck Millipore, Darmstadt, Germany) to detect neurons. In order to perform immunohistochemistry experiments, animals were anesthetized and transcardially perfused with 0.1M phosphate buffer (PB) followed by 4% paraformaldehyde. Brains were then removed and postfixed in the same solution for 4 h and then cryoprotected in 30% v/v sucrose solution. Finally, brains were sliced into 35 µm coronal sections using a microtome. In addition, staining tissue was previously treated with 2N HCl for 30 min at 37°C after fixation and then neutralized in tap water. Sections were mounted using Fluoroshield (Sigma Aldrich, Madrid, Spain).

2.10 Western Blot

Mice were sacrificed and PFC and hippocampal regions were dissected and stored at -80°C. Brain areas were homogenized in lysis buffer containing 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1% Triton X-100 with one protease inhibitor tablet (complete ULTRA Protease Inhibitor Cocktail Tablets, 05892970001 Roche) and one phosphatase inhibitor tablet (PhosSTOP Inhibitor Cocktail Tablets, 04906845001, Roche) per 10 ml of buffer. Then, homogenates (20

µg of total protein) were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS/PAGE) before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad, Spain). Membranes were blocked in Tris-buffered saline (TBS) (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk for 1 h and then incubated with rabbit anti-Synapsin I (1:1000, Cat. Ab64581, Abcam), rabbit anti-Calceineurin A (1:1000, Cat. Ab3673, Abcam) or mouse anti-β-Tubulin (1:5000, Cat 556321, BD Pharmingen) for 4 h. Finally, membranes were incubated with secondary fluorescent antibodies, anti-mouse (1:2500, Cat. 610-132-121, IRDye 800, Rockland) and anti-rabbit (1:2500, Cat. 611-144-002-0.5, IRDye 680, Rockland) for 1 h. Visualization and quantification were carried out using the LI-COR Odyssey® scanner and software (LI-COR Biosciences). Protein densities were normalized to the detection of the housekeeping control β-Tubulin in the same samples and expressed as a percentage of the control group.

2.11 Preparation and analysis of total synaptosomal membranes and subsynaptic fractionation

Hippocampus and PFC from 6 mice were homogenized at 4°C in 1 mL of isolation buffer containing 0.32 M sucrose, 0.1 mM CaCl₂ and 0.1 mM MgCl₂, pH 7.4. The resulting homogenate was mixed with 6 mL sucrose (2 M) and 2.5 mL CaCl₂ 0.1 (mM) in a centrifuge tube (Ultra-Clear Beckman Coulter). Subsequently, 2.5 mL of sucrose (1 M) containing 0.1 mM CaCl₂ were slowly added to form a sucrose gradient. Following centrifugation for 3 h at 100,000 x g at 4°C, the synaptosomes were collected as the interphase between 1.25 M and 1 M sucrose. They were diluted 10 times in isolation buffer, centrifuged for 30 min at 15,000 x g at 4°C and the resulting synaptosomal pellet was resuspended in 1 mL of isolation buffer for immediate use.

The separation of the presynaptic active zone, postsynaptic density and extrasynaptic fractions from hippocampal and cortical synapses was carried out as previously described (Phillips et al., 2001). Briefly, synaptosomes were diluted 1:10 in cold 0.1 mM CaCl₂ and an equal volume of 2x solubilisation buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The suspension was incubated for 30 min on ice with agitation and the insoluble material (synaptic junctions) pelleted (40,000 x g for 30 min at 4°C). The supernatant (extrasynaptic fraction) was concentrated using

an Amicon Ultra 15 10K and proteins precipitated with six volumes of acetone at -20°C and recovered by centrifugation (18,000 x g for 30 min at -15°C). The synaptic junctions pellet was washed in solubilisation buffer (pH 6.0) and resuspended in 10 volumes of a second solubilisation buffer (1% Triton X-100, 20 mM Tris at pH 8.0). After incubation for 30 min on ice with agitation, the mixture was centrifuged and the supernatant (pre-synaptic fraction) processed as described for the extrasynaptic fraction, whereas the insoluble pellet corresponds to the postsynaptic fraction. Protease inhibitors (Protease Inhibitor Cocktail Set III, Millipore, Temecula, CA, USA) were added to the suspension in all extraction steps. The protein concentration determined by the bicinchoninic acid protein assay (Pierce), and 20 µg of each fraction, solubilized in 5% SDS, were added to SDS-PAGE sample buffer prior to freezing at -20°C. SDS/PAGE was performed using 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA) and immunoblotted with the indicated antibody (rabbit anti-synaptophysin antibody, Cat. ab52636, Abcam; mouse anti-SNAP25 antibody, Cat. ab66066, Abcam; and mouse anti-PSD95 antibody, Cat. ab2723, Abcam), and then with a horseradish peroxidase (HRP)-conjugated corresponding secondary antibody. The immunoreactive bands were developed using a chemiluminescent detection kit (Thermo Fisher Scientific, Inc.) and detected with an Amersham Imager 600 (GE Healthcare Europe GmbH, Barcelona, Spain).

2.12 Statistical analysis

All statistical analyses were performed using GraphPadPrism 6 (GraphPad Software, San Diego, CA). Data from electric nociceptive threshold (locomotor activity and number of jumpings), and immunohistochemistry were analysed using an unpaired two-tailed Student's t-test. Data from electric nociceptive threshold (vocalizations) were analysed using a Chi-square test. Data from Y-maze, radial arm maze, active and passive avoidance paradigms, and western blot were analysed using two-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test when required. The data are expressed as mean ± SEM, and statistical significance was set at $p < 0.05$.

3. Results

3.1 Working memory alterations in adult mice lacking A_{2A}R in adult mice

Since A_{2A}R function has been associated to memory function (Cunha et al., 2008; Fontinha et al., 2009), we first evaluated the impact of A_{2A}R and age progression in working memory processing in mice. For that, we used the spontaneous alternation Y maze test. Interestingly, two-way ANOVA showed an effect of age [$F_{(1,53)} = 6.903$, $p < 0.05$] and genotype [$F_{(1,53)} = 12.09$, $p < 0.01$], but no interaction between both factors [$F_{(1,53)} = 1.419$, n.s.]. The Bonferroni post-hoc test showed differences between adults WT and A_{2A}R KO mice ($p < 0.01$) (Fig 1).

3.2 Deletion of A_{2A}R leads to deficits in spatial memory

Next, we aimed to evaluate the impact of A_{2A}R expression in spatial memory using the radial arm maze in which mice were trained for 19 consecutive days. Interestingly, the deletion of A_{2A}R impaired spatial memory as revealed two-way ANOVA, that showed an effect of genotype [$F_{(1,15)} = 12.95$, $p < 0.01$], time [$F_{(18,270)} = 2.064$, $p < 0.01$] and interaction between both factors [$F_{(18,270)} = 1.787$, $p < 0.05$], thus indicating a different acquisition pattern between WT and A_{2A}R KO mice (Fig 2A).

The study of such effects in middle-aged mice led to similar results (Fig 2B). Statistical analysis showed significant effects of genotype [$F_{(1,29)} = 16.60$, $p < 0.001$], time [$F_{(18,522)} = 5.339$, $p < 0.001$] and interaction between both factors [$F_{(18,522)} = 2.068$, $p < 0.01$], thus also indicating differential learning patterns between WT and A_{2A}R KO mice. The Bonferroni post-hoc test showed a difference in the learning process between WT and A_{2A}R KO mice in the following days: 8 ($p < 0.05$), 11 ($p < 0.001$) and 12 ($p < 0.01$).

3.3 A_{2A}R deletion leads to minimal changes in electric pain sensitivity

In order to assess the initial behavioural reactivity to electric foot-shocks, we measured the electric nociceptive threshold in adult mice. Statistical analysis showed no differences in locomotor activity (number of rearings and squares crossed) during the habituation period ($t = 0.429$, $df = 7$, n.s.) and during the test ($t = 0.748$, $df = 7$, n.s.) respectively, and no changes were observed in vocalizations. However, A_{2A}R

KO mice jumped less than their WT littermates ($t = 2.537$, $df = 7$, $p < 0.05$) showing slight differences in pain sensitivity between genotypes (Table 1).

3.4 A_{2A}R deletion leads to emotional learning deficits in the passive avoidance test in adult mice

In order to assess differences in emotional memory in response to the deletion of A_{2A}R, we conducted the passive avoidance test at adulthood and middle-aged age. In the adult group, two-way ANOVA revealed effect of genotype [$F_{(1,32)} = 6.584$, $p < 0.05$], day [$F_{(1,32)} = 8.919$, $p < 0.05$] and interaction between both factors [$F_{(1,32)} = 5.563$, $p < 0.01$]. Bonferroni post-hoc analysis showed differences in the WT between the acquisition and retention trial ($p < 0.01$), and between genotypes in the retention trial ($p < 0.01$), indicating deficits in associative learning in A_{2A}R KO animals (Fig 3A).

However, in the middle-aged group of mice, two-way ANOVA showed effect of day [$F_{(1,58)} = 34.23$, $p < 0.001$] with no effect of genotype [$F_{(1,58)} = 0.408$, n.s.] or interaction between both factors [$F_{(1,58)} = 0.004$, n.s.] (Figure 3B).

3.5 A_{2A}R deletion induces overall associative learning impairments in the active avoidance test.

To study associative learning using a more demanding protocol, we used active avoidance paradigm. In adults, two-way ANOVA of conditioned responses showed effect of time [$F_{(9,279)} = 42.51$, $p < 0.001$] and genotype [$F_{(1,31)} = 10.79$, $p < 0.01$], but no interaction between both factors [$F_{(9,279)} = 1.679$, n.s.] (Fig 4A). The analysis of unconditioned responses also showed an effect of time [$F_{(9,279)} = 41.71$, $p < 0.001$] and genotype [$F_{(1,31)} = 10.74$, $p < 0.01$], but no interaction between both factors [$F_{(9,279)} = 1.724$, n.s.] (Fig 4B).

In middle-aged mice, two-way ANOVA of conditioned responses showed effect of time [$F_{(9,270)} = 28.28$, $p < 0.001$] and genotype [$F_{(1,30)} = 6.366$, $p < 0.05$], but no interaction between both factors [$F_{(9,270)} = 1.568$, n.s.] (Fig 4C). The study of unconditioned responses also showed effect of time [$F_{(9,270)} = 27.86$, $p < 0.001$] and genotype [$F_{(1,30)} = 6.296$, $p < 0.05$], but no interaction between both factors [$F_{(9,270)} = 1.553$, n.s.] (Fig 4D).

3.6 Lack of A_{2A}R leads to decreased neurogenesis in adult mice

The study of neurogenesis in adult A_{2A}R mice showed that transgenic mice have a reduction in newborn neurons ($t = 2.869$, $df = 8$, $p < 0.05$) as seen after the 3-day sacrifice (Fig 5A and 5B). However, no changes were observed after 30 days in the survival study ($t = 0.581$, $df = 6$, n.s.), suggesting that once born, neurons had the same survival rates (Fig 5B).

3.7 A_{2A}R deletion leads to changes in calcineurin and synapsin expression

The study of learning and memory related proteins in adult mice using a two-way ANOVA showed for synapsin no effect of genotype [$F_{(1,19)} = 0.6095$, n.s.], but an effect of age [$F_{(1,19)} = 5.497$, $p < 0.05$] and interaction [$F_{(1,19)} = 5.497$, $p < 0.05$] between factors. Bonferroni post-hoc test showed differences between adult and middle-aged A_{2A}R KO mice ($p < 0.05$) (Fig 6). In the case of calcineurin levels, two-way ANOVA showed an effect of genotype [$F_{(1,20)} = 13.88$, $p < 0.01$] but no effect of age [$F_{(1,20)} = 0.01461$, n.s.] neither interaction [$F_{(1,20)} = 0.01461$, n.s.] between factors (Fig. 6).

3.8 A_{2A}R deletion leads to changes in synaptic protein expression

As our behavioural results pointed to age-related cognitive impairments in the A_{2A}R KO mice, we then aimed to assess whether they correlated with changes in the expression of synaptic proteins. To this end, we performed subsynaptic fractionation of hippocampal and PFC nerve terminals from adult and aged WT and A_{2A}R KO mice, which allowed us to identify the expression levels of extra-, pre- and postsynaptic enriched proteins (i.e. synaptophysin, SNAP25 and PSD95, respectively) (Fig 7). Interestingly, these immunoblots revealed an increase in the expression of synaptophysin in the A_{2A}R KO hippocampus and PFC both in adult and middle-aged mice (Fig 7A and C). Accordingly, when synaptophysin immunoreactivity was semi-quantified in the extrasynaptic fraction, a significant increase was observed in the PFC of A_{2A}R KO when compared to WT mice [$F_{(1,8)} = 17.85$, $p < 0.01$] and also when middle-aged mice were compared to adult animals [$F_{(1,8)} = 5.85$, $p < 0.05$] (Fig 7B and D). Also, the postsynaptic protein marker PSD95 showed a similar significant increase in its expression in the hippocampus [$F_{(1,8)} = 10.04$, $p < 0.05$] and the PFC [$F_{(1,8)} = 10.77$, $p < 0.05$] of the A_{2A}R KO when compared to WT mice (Fig 7). Importantly, the presynaptic protein marker SNAP25 showed a significant reduction

in its expression in the hippocampus ($t = 3.702$, $df = 8$, $p < 0.05$) and PFC ($t = 7.136$, $df = 8$, $p < 0.001$) of adult A_{2A}R KO when compared to the same aged WT mice (Fig 7). In addition, while in aged animals the PFC SNAP25 was significantly reduced ($t = 9.511$, $df = 8$, $p < 0.001$), in the hippocampus a significant increase was observed ($t = 2.837$, $df = 8$, $p < 0.05$), thus suggesting a differential age-related regulation of SNAP25 expression between these two brain areas. Overall, an altered expression of subsynaptic components would seem to correlate with age-related cognitive deficits.

4. Discussion

In this study, we evaluated the effects of selective A_{2A}R deletion in the regulation of cognitive processes. Under our experimental conditions we showed overall cognitive impairments in A_{2A}R KO mice associated with a decrease in new-born neuron proliferation. We also observed a reduced expression of calcineurin and synapsin in the PFC of A_{2A}R KO mice and calcineurin in the PFC of middle-aged A_{2A}R KO mice, in addition to changes in extra-, pre- and post-synaptic markers between WT and A_{2A}R KO mice.

Cognitive impairments have been observed in many psychiatric diseases and often have a significant impact on the functional outcome of the disease (Kumar and Frangou, 2010; Millan et al., 2012). In schizophrenia, executive function deficits have been described in patients and also in first-degree relatives (Breton et al., 2011), and spatial and emotional deficits have also been observed (Hall et al., 2007; Spieker et al., 2012). Clinical studies have also shown significant cognitive deficits and retardation affecting learning and memory processes in depressed patients (Eriksson et al., 2012). Moreover, cognitive functions were impaired in patients affected of bipolar disorders and unaffected first-degree relatives (Arts et al., 2008; Glahn et al., 2010). Our study suggests that the inactivation of the A_{2A}R induces overall cognitive deficits including impairments in functional prefrontal cortex integrity, in addition to spatial and associative memory in A_{2A}R KO mice. However, a few of these findings contradict some of the previous results relating to the role of A_{2A}R in cognition. While some studies have found that agonists impair memory retrieval in rats (Pereira et al., 2005), others show cognitive enhancement in A_{2A}R KO mice (Shen et al., 2012) or after treatment with specific A_{2A}R antagonists (Takahashi, 2008), and some

suggest that the activation of adenosine A_{2A}R is sufficient to induce cognitive impairments in mice (Pagnussat et al., 2015). On the contrary, other studies have shown alterations in the active avoidance paradigm in striatum-specific A_{2A}R KO mice (Singer et al., 2013), and the deletion of adenosine A_{2A}R induced changes in episodic memory assessed with the novel object recognition test (Moscoso-Castro et al., 2016). The selective inactivation of A_{2A}R in astrocytes also impairs working memory in mice (Matos et al., 2015). Importantly, several articles have pointed the role of adenosine A_{2A}R in synaptic plasticity. Indeed, the blockade of A_{2A}R abolishes long-term potentiation (Fontinha et al., 2009) and, in A_{2A}R KO mice, a reduction in the induction of long-term potentiation in the nucleus accumbens may be observed (d'Alcantara et al., 2001), highlighting the role of A_{2A}R in the control of learning and memory processes.

Under our experimental conditions, we observed mild changes relating to jumping in the electric nociceptive threshold (Table 1). In accordance with such changes, the first characterization of CD1-background adenosine A_{2A}R KO mice showed hypoalgesia in response to a thermal stimulus (Ledent et al., 1997). Although we cannot exclude changes in pain sensitivity to mediate the alterations observed in the passive and active avoidance, alterations observed in pain threshold were minimal and no differences were observed in the number of vocalizations.

Knowledge is limited as to the course of cognitive symptoms in psychiatric disorders. In schizophrenia for example, patients tend to present a heterogeneous but commonly deteriorating clinical course (Lieberman, 1999), although the few longitudinal studies available provide, in some cases, contradictory results (Davidson et al., 1995; Harvey et al., 1992; 1997; 1999). Depression has also been associated with cognitive impairments and dementia (Ganguli, 2009). In the case of bipolar disorder, three longitudinal studies of cognitive function showed no major changes in age-related cognition (Balanzá-Martínez et al., 2005; Engelsmann et al., 1988; Sarnicola et al., 2009). We also therefore performed the abovementioned cognitive battery test in 12-month old mice to assess the cognitive performance of WT and A_{2A}R KO mice with age. Our results show that A_{2A}R KO middle-aged mice display deficits in spatial memory and associative memory, as observed in the radial arm maze test and the active avoidance paradigm. However, some of the cognitive deficits observed in adult

mice are lost with age. This may be due to a ceiling effect in aged mice, as performance in cognitive tests worsened both in WT and A_{2A}R KO mice.

Many psychiatric disorders emerge during adolescence mainly due to aberrations in normal developmental and maturation of brain processes (Paus et al., 2008). In fact, there are several factors influencing cognitive processes which occur during neural development, including synaptogenesis and neurogenesis, pruning, myelination and cortical thinning (Tau and Peterson, 2010). Several of these processes are indeed strongly affected in schizophrenia, depression, bipolar disorder and ADHD (Alexander-Bloch et al., 2014; Paus et al., 2008; Stanley et al., 2008). Neurogenesis is not limited to neurodevelopment, and may be observed in adulthood in the DG, the subventricular zone and the olfactory bulb (Ming and Song, 2011). In rodents, adult neurogenesis can be determined using the exogenous cell tracer BrdU, a thymidine analogue that incorporates into dividing cells during DNA synthesis, thus enabling the visualization of new-born neurons (Wojtowicz and Kee, 2006). Although there are few data as to the effects of adenosine on neurogenesis, the activation of A₁R has been found to inhibit neurogenesis in the subventricular zone (Benito-Muñoz et al., 2016), suggesting that exogenous adenosine may contribute to the regulation of neurogenesis. Under our experimental conditions we found that the deletion of adenosine A_{2A}R in adult mice impaired the proliferation of new-born neurons, although neuron survival was not affected.

Some of these psychiatric diseases, especially schizophrenia, may be considered as a connectivity disorder, suggesting the appearance of abnormalities in plasticity (Friston, 2002; Schmitt et al., 2011). One way to assess synaptic connectivity would be to study the expression of proteins essential for synaptic function in brain areas that have shown cognitive deficits, such as the PFC and hippocampus. Synaptic vesicle proteins such as synapsin regulate and/or mediate the storage and release of neurotransmitters and thus play a prominent role in synaptic transmission and plasticity (Fdez and Hilfiker, 2006). In neuropsychiatric diseases, authors have also described the presence of abnormal density and morphology of dendritic spines, synapse loss, and aberrant synaptic signalling and plasticity, which may be defined as synaptic pathology (Blanpied and Ehlers, 2004; van Spronsen and Hoogenraad, 2010). Indeed, decreased synapsin expression has been widely studied in

neurodevelopmental diseases such as schizophrenia and has been postulated as a marker of synaptic pathology (Eastwood et al., 1995). Moreover, mice lacking synapsin present learning and memory deficits (Corradi et al., 2008; Schmitt et al., 2009), highlighting the role of these proteins in cognitive processes. Finally, in addition to synapsin, we also evaluated the expression of calcineurin. Calcineurin is a calcium-dependent protein phosphatase which acts as a major regulator of key proteins essential for synaptic transmission and neuronal excitability (Baumgärtel and Mansuy, 2012). Forebrain-specific calcineurin deletions lead to deficits in spatial and working/ episodic memory (Zeng et al., 2001) and calcineurin-KO mice have been proposed as an animal model of schizophrenia (Cottrell et al., 2013; Miyakawa et al., 2003). Under our experimental conditions, we found that synapsin levels changed differentially with age and while a tendency to decrease was found in adult A_{2A}R KO mice, middle-aged mice showed the contrary tendency. In the case of calcineurin, a decrease in the PFC of both adult and middle-aged A_{2A}R KO mice was found. Interestingly, no studies to date have examined calcineurin protein levels in the genetic or pharmacological blockade of A_{2A}R.

Subcellular fractionation of PFC nerve terminals revealed a reduction of the presynaptic marker SNAP25 in A_{2A}R KO mice, thus supporting the notion of a loss of PFC neuronal connectivity upon A_{2A}R deletion (Ramírez et al., 2004). Interestingly, the SNAP25 expression decline was more pronounced in the aged mice than in adults, thus indicating a higher sensitivity to synaptic loss along A_{2A}R KO mouse aging. Remarkably, while a similar decline in hippocampal SNAP25 expression was observed in adult A_{2A}R KO mice, in the hippocampus of middle-aged mice an increased expression was confirmed. Thus, such results would suggest a differential age-related regulation of synaptic connectivity between these two brain areas. Interestingly, it has been reported that patients with schizophrenia show a considerable reduction of SNAP25 immunoreactivity in the PFC and hippocampus (Thompson et al., 2003), in line with our synapsin and calcineurin results. Conversely, when the postsynaptic marker PSD95 was analysed, an increased expression in adult and aged A_{2A}R KO mice was always observed. Notably, it has been reported that PSD95 expression is significantly reduced in the PFC, but not in the hippocampus, of neuroleptic-treated schizophrenics (Ohnuma et al., 2000). Thus, our results in the A_{2A}R KO mice differ from these described in schizophrenic patients, which may be

related to the chronic neuroleptic treatment of patients. Interestingly, our findings lead us to hypothesize that the increased PSD95 expression in the A_{2A}R KO mice may be the consequence of a compensatory mechanism in response to synaptic loss, as described for okadaic acid-induced neuritic retraction (Leuba et al., 2008).

Consequently, such data implicate deficient adenosine signalling in cognitive processes indicating the presence of synaptic pathology after the selective inactivation of A_{2A}R, providing new possibilities for the therapeutic management of the cognitive deficits associated with psychiatric disorders.

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Table 1. Effects of A_{2A}R deletion on electric foot-shock reactivity.

	Locomotor activity		Electric foot-shock reactivity	
	Rearings	Squares crossed	Jumping	Vocalizations
WT	74,75 ± 4,67	176,75 ± 12,47	44,5 ± 3,8	1 ± 0
A _{2A} R KO	78 ± 6,18	189,5 ± 7,83	33,87 ± 1,75*	1 ± 0

Data are expressed as mean ± SEM of the number of behavioural parameters observed, N = 10 mice per group. * p<0.05, genotype effect (two-tailed Student's t-test).

Legends of the Figures

Figure 1. Effects of A_{2A}R deletion on the spontaneous Y maze test. Mean $\pm$ SEM percentage of alternations in the Y-maze made by **A)** adult and **B)** middle-aged WT and A_{2A}R KO mice (n = 14-15). ** p<0.01, genotype effect (Two-way ANOVA and Bonferroni post-hoc test).

Figure 2. Effects of A_{2A}R deletion on the radial arm maze. Reference memory errors in **A)** adult (n = 10) and **B)** middle-aged (n = 15-20) mice, respectively. Values are expressed as the mean $\pm$ SEM. ** p<0.01, *** p<0.001, genotype effect (Two-way ANOVA, Bonferroni post-hoc test).

Figure 3. Effects of A_{2A}R deletion on the passive avoidance test. Latency time in entering the dark compartment in acquisition and retention trials in **A)** adult, and **B)** middle-aged mice were evaluated. Data are expressed as mean $\pm$ SEM of the latency time, N = 9 mice per group at adulthood, and N = 14–15 mice per group in aged mice. ** p<0.01 genotype effect, ## p<0.01, ### p<0.001 trial effect (Two-way ANOVA, Bonferroni post-hoc test).

Figure 4. Effects of A_{2A}R deletion on the active avoidance paradigm. **A), C)** conditioned responses, **B), D)** unconditioned responses in adult (n = 10) and middle-aged (n = 15-20) mice, respectively. Values are expressed as the mean $\pm$ SEM. * p<0.05, ** p<0.01, genotype effect (Two-way ANOVA, Bonferroni post-hoc test).

Figure 5. Effect of A_{2A}R deletion on neuronal progenitor's proliferation and survival. **A)** Images of BrdU and NeuN immunostaining. Data are presented as mean $\pm$ SEM of BrdU-positive cells in **B)** proliferation and survival studies of WT and A_{2A}R KO mice (n=5). *p<0.05 genotype comparison (two-tailed Student's t-test).

Figure 6. Effect of A_{2A}R deletion on protein expression. **A)** Representative western blot and densitometry quantification of **B)** synapsin and **C)** calcineurin protein expression levels in the PFC of adult and aged mice, respectively. Data are presented

as mean $\pm$ SEM (n=5-6 per group). *p<0.05 genotype comparison (Two-way ANOVA and Bonferroni post-hoc test).

Figure 7. Characterization of the subsynaptic distribution of synaptic markers. Representative western blot (**A, C**) and densitometry quantification (**B, D**) of the synaptophysin, SNAP25 and PSD95 in hippocampal region (**A, B**) and PFC region (**C, D**) of mouse brains. Mice were divided into four different groups according to genotype (WT, A_{2A}R KO) and age (adult, middle-aged). N=4-5 per group, *p<0.05, **p<0.01, ***p<0.001 two-way ANOVA, Bonferroni post-hoc test. The Y-axis represents arbitrary units.