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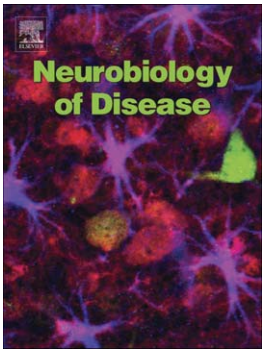


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PII: S0969-9961(15)00127-8
DOI: doi: 10.1016/j.nbd.2015.03.030
Reference: YNBDI 3489

To appear in: *Neurobiology of Disease*

Received date: 27 October 2014
Revised date: 18 March 2015
Accepted date: 31 March 2015

Please cite this article as: Li, Wei, Silva, Henrique B., Real, Joana, Wang, Yu-Mei, Rial, Daniel, Li, Ping, Payen, Marie-Pierce, Zhou, Yuanguo, Muller, Christa E., Tomé, Angelo R., Cunha, Rodrigo A., Chen, Jiang-Fan, Inactivation of adenosine $A_{2A}$ receptors reverses working memory deficits at early stages of Huntington's disease models, *Neurobiology of Disease* (2015), doi: 10.1016/j.nbd.2015.03.030

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Re-revised MS submitted to "*Neurobiology of Disease*"

March 17, 2015

# Inactivation of adenosine $A_{2A}$ receptors reverses working memory deficits at early stages of Huntington's disease models

Running title: $A_{2A}$ receptors control cognition in HD

Wei Li[1,2*], Henrique B. Silva[3*], Joana Real[3*], Yu-Mei Wang[1], Daniel Rial[3], Ping Li[1,4], Marie-Pierce Payen[1],Yuanguo Zhou[4], Christa E. Muller[6], Angelo R. Tomé[3], Rodrigo A. Cunha[3,5], Jiang-Fan Chen[1]

[1]Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA; [2]Department of Neurology, Research Institute of Surgery and Daping Hospital, Third Military Medical University, Chongqing, China; [3]CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Portugal; [4]Molecular Biology Center, State Key Laboratory of Trauma, Burn, and Combined Injury, Research Institute of Surgery and Daping Hospital, Third Military Medical University, Chongqing, China; [5]FMUC-Faculty of Medicine, University of Coimbra, Portugal.

* these authors contributed equally to this work.

Address correspondence: Jiang-Fan Chen, Department of Neurology, Boston University, School of Medicine, Boston 02129, MA, USA. Tel: +1 6174141249; Email: chenjf@bu.edu.

Number of figures: 6 Number of words: abstract, 241; Introduction, 489; Discussion, 1,943

Conflict of interest: All authors disclose no conflict of interest.

**Acknowledgements**: We thank Caroline Veloso for technical support. This study was supported by the CHDI foundation, NIH grant R01NS073947 and NS041083-07, Santa Casa da Misericordia, DARPA and FCT (PTDC/SAU-TOX/122005/2010 and PEST-C/SAU/LA0001/2013-14).

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**Abstract**

Cognitive impairments in Huntington's disease (HD) are attributed to a dysfunction of the cortico-striatal pathway and significantly affect the quality of life of the patients, but this has not been a therapeutic focus in HD to date. We postulated that adenosine $A_{2A}$ receptors ($A_{2A}R$), located at pre- and post-synaptic elements of the cortico-striatal pathways, modulate striatal neurotransmission and synaptic plasticity and cognitive behaviors. To critically evaluate the ability of $A_{2A}R$ inactivation to prevent cognitive deficits in early HD, we cross-bred $A_{2A}R$ knockout (KO) mice with two R6/2 transgenic lines of HD (CAG120 and CAG240) to generate two double transgenic R6/2-CAG120-$A_{2A}R$ KO and R6/2-CAG240-$A_{2A}R$ KO mice and their corresponding wild-type (WT) littermates. Genetic inactivation of $A_{2A}R$ prevented working memory deficits induced by R6/2-CAG120 at postnatal week 6 and by R6/2-CAG240 at postnatal month 2 and postnatal month 3, without modifying motor deficits. Similarly the $A_{2A}R$ antagonist KW6002 selectively reverted working memory deficits in R6/2-CAG240 mice at postnatal month 3. The search for possible mechanisms indicated that the genetic inactivation of $A_{2A}R$ did not affect ubiquitin-positive neuronal inclusions, astrogliosis or Thr-75 phosphorylation of DARPP-32 in the striatum. Importantly, $A_{2A}R$ blockade preferentially controlled long-term depression at cortico-striatal synapses in R6/2-CAG240 at postnatal week 6. The reported reversal of working memory deficits in R6/2 mice by the genetic and pharmacological inactivation of $A_{2A}R$ provides a proof-of-principle for $A_{2A}R$ as novel targets to reverse cognitive deficits in HD, likely by controlling LTD deregulation.

**Key words**: adenosine $A_{2A}$ receptor, Huntington's disease, cognition, working memory, long-term depression, R6/2 mice

**Abbreviations**: $A_{2A}R$= adenosine $A_{2A}$ receptor; aCSF= artificial cerebrospinal fluid; DARPP-32= dopamine- and cAMP-regulated phosphoprotein with molecular weight of 32 kDa; HD= Huntington's disease; KO= knock out; LTD= long-term depression; LTP= long-term potentiation; PN= postnatal day; WT= wild type.

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## Introduction

Huntington's disease (HD) is characterized by progressive motor, cognitive, and psychiatric symptoms (Zuccato et al., 2010). The largest and most comprehensive study of HD cognitive impairment (PREDICT-HD study, including 738 HD prodromes) (Stout et al., 2011) and several others (Duff et al., 2010; Lawrence et al., 1998; Papp et al., 2011) have conclusively demonstrated cognitive impairments in the HD prodrome, 5-15 years before the onset of motor symptoms. Cognitive impairment in early HD patients is characterized by perseverative behavior, reduced reversal learning and working memory deficits (Giralt et al., 2012; Papp et al., 2011). Similarly, transgenic HD (R6/2 and YAC128) (Brooks et al., 2006; Brooks et al., 2012; Lione et al., 1999; Mazarakis et al., 2005; Van Raamsdonk et al., 2005) and knock-in (KI) mouse lines (Brooks et al., 2006; Trueman et al., 2009) display impairments of reversal learning and working memory. These cognitive changes herald a primary dysfunction of the cortico-striatal pathway (Cepeda et al., 2010; Cepeda et al., 2007), and significantly affect the patient's quality of life, but this has not been a therapeutic focus in HD to date. We postulate that striatal adenosine $A_{2A}$ receptors ($A_{2A}R$) at pre- and post-synaptic elements of cortico-striatal synapses control cortico-striatal function and reverse cognitive inflexibility in early HD. While *presynaptic* $A_{2A}R$ at corticostriatal glutamatergic terminals (Rebola et al., 2005a; Rosin et al., 2003) strategically modulate glutamate release (Ciruela et al., 2006; Rodrigues et al., 2005), *postsynaptic* striatal $A_{2A}R$ physically and functionally interact antagonistically with dopamine $D_2$ and NMDA receptors and synergistically with metabotropic glutamate 5 and cannabinoid CB1 receptors (reviewed in Schiffmann et al., 2007). $A_{2A}R$ at the corticostriatal pathway can fine-tune striatal synaptic plasticity by modulating long-term plasticity at cortico-striatal synapses (d'Alcantara et al., 2001; Flajolet et al., 2008; Shen et al., 2008), thus potentially controlling the glutamatergic hyperactivity at cortico-striatal synapses in early HD (Hong et al., 2012; Joshi et al., 2009; Klapstein et al., 2001). Consistent with the ability of $A_{2A}R$ to control striatal plasticity, we have shown that genetic inactivation of $A_{2A}R$ enhances working memory (Augusto et al., 2013; Wei et al., 2011; Zhou et al., 2009), reversal learning (Wei et al., 2011) and goal-oriented behavior (Yu et al., 2009). Furthermore, pharmacological blockade of $A_{2A}R$ prevents memory impairment in models of Alzheimer's disease (Canas et al., 2009; Cunha et al., 2008),

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Parkinson's disease (Kadowaki Horita et al., 2013) and aging (Prediger et al., 2005). Thus, $A_{2A}R$ emerge as promising therapeutic targets to reverse cognitive deficits in HD.

To critically evaluate the ability of $A_{2A}R$ inactivation to reverse cognitive deficits in HD, we cross-bred $A_{2A}R$ knockout (KO) mice with two R6/2 transgenic lines of HD (CAG120 and CAG240) to generate two double transgenic R6/2-CAG120-$A_{2A}R$ KO and R6/2-CAG240-$A_{2A}R$ KO mice. We focused on the ability of $A_{2A}R$ inactivation to reverse cognitive deficit (specifically working memory) and synaptic plasticity at early stages of HD, making it a more plausible therapeutic strategy without the need to consider reversing striatal neurodegeneration.

## Materials and methods

### *Generation of two double transgenic R6/2-CAG120 or R6/2-CAG240 x $A_{2A}R$ KO lines*

Double transgenic R6/2-$A_{2A}R$ KO mice were generated by crossing congenic global-$A_{2A}R$ KO mice in C57BL/6 background (Chen et al., 1999) with R6/2-CAG120 or with R6/2-CAG240 mice. We first cross-bred $A_{2A}R$ KO with R6/2-CAG120 lines (in mixed C57BL6 x B6CBA genetic background) taking advantage of the clear experimental endpoints provided by this R6/2-CAG120 line (Gil and Rego, 2009; Menalled and Chesselet, 2002). Thus, R6/2-CAG120 mice develop cognitive deficits starting at post-natal day (PN) 35, prior to the onset of locomotor deficits, which are evident around PN40 (Carter et al., 1999; Lione et al., 1999). Since this provides a short window to study cognitive deficits without motor confounding effects, we next explored R6/2-CAG240 mice (in congenic C57BL6 genetic background), which exhibits cognitive deficits at postnatal month 2, prior to motor symptoms. Importantly, we have adapted an ovarian transplant strategy to increase our ability to generate sufficient large cohorts of the double transgenic lines. Animals were maintained in a controlled environment (23±2°C; 12 h light/dark cycle; *ad libitum* access to food and water). All studies and manipulations of animals were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, FELASA and ARRIVE and were authorized by the Animal Care and Use Committee at Boston University School of Medicine and the Ethical Committee of the Center for Neuroscience and Cell Biology of the

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University of Coimbra.

***Treatment with the $A_{2A}$R antagonist KW6002***

KW6002 was synthesized by Christa Muller's lab (Bonn University, Germany), as previously described (Hockemeyer et al., 2004). KW6002 was dissolved in a fresh mixture of dimethylsulfoxide (15%) and ethoxylated castor oil (15%; Alkamuls, EL-620, Rhodia) and was injected (10 mL/kg) intra-peritoneally at a dose of 1 mg/kg, 30 min prior to behavioral testing.

***Behavioral analyses***

*Motor coordination:* After familiarization to the rotarod apparatus (Columbus Instruments) during 2 day, mice were placed on a rod that was rotated at accelerating speed from 4-40 rpm. Each mouse was given three trials for a maximum of 300 sec for each trial. The time taken by each mouse to fall off the rotating rod was recorded and used as an index of motor coordination.

*Reference and working memory*: We evaluated working memory and short-term reference using the 8-arm radial maze, as previously described (Augusto et al., 2013; Zhou et al., 2009). The 8-arm radial maze consists of an octagonal platform and eight radial arms, which were extending outwards from the platform (San Diego Instrument). First, a food deprivation schedule was carried out in order to reduce the animal's weight to 85% of the baseline. Next, an acclimation trial was conducted: on day 1, food pellets were scattered throughout the arms and platform, and mice were allowed to freely eat the food in the maze; on day 2, food pellets were placed in the arms only; on day 3, food pellets were located on the food cup. We then started the testing period in the next 5 days. Each day, food pellets were placed in the cups of only 4 arms (*e.g.* arms #1, #2, #4 and #7) while the remaining 4 arms were empty. At the beginning of each trial, the mouse was placed in the central platform and allowed to explore the maze. When the four food pellets were eaten or 5 min is elapsed, a trial was completed. A working memory error is defined when an animal revisits an arm of the maze that was just entered in the course of collecting all 4 rewards while a reference memory error is defined when the animal visits a non-baited arm (arms #3, #5, #6 and #8).

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Because some of the mice failed to eat all of the food pellets on the first testing day (day 1), we recorded the mouse performance from the second testing day (day 2).

To exclude the possible confounding effect of alterations of locomotor activity after KW6002 treatment (1 mg/kg), we used the frequency of entrance into the arms (entrances/10 seconds) as an indirect indicator of the general locomotor activity.

*Survival analysis*: Mice were observed twice daily, in the morning and later afternoon, and weighted twice a week at the same time. The criteria for euthanasia were: (1) decrease of body weight of more than 20% within one week, (2) inability to obtain food and water, (3) difficulties in breathing and inability to move. These end points were considered equivalent to the time of death.

***Immunohistochemistry analyses***

At the end of behavioral testing, the mice were anesthetized with Avertin and perfused transcardially with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed for 1 h, rinsed in 0.1 M phosphate buffered saline, and then cryoprotected in a graded series of 10% and 20% glycerol with 2% dimethylsulfoxide solution. Frozen brains were sectioned at 30 μm using a sledge microtome. The sections were processed for immunohistochemistry of astrogliosis (GFAP immunoreactivity), ubiquitin-positive neuronal intra-nuclear inclusions and DARPP-32 phosphorylation at Thr-75 focusing in the striatum, as previously described (Rebola et al., 2011; Shen et al., 2013), to correlate these neurochemical changes with the working memory deficits in R6/2 mice and their reversal by $A_{2A}R$ inactivation. Free-floating sections were incubated for 30 min in 0.3% Triton-X100 and 3% normal donkey or goal serum. Incubations with primary antibodies against glial fibrillary acidic protein (GFAP, Millipore, 1:1000), ubiquitin (Dako, 1:100), or DARPP-32-phospho-threonine75 (Cell Signal, 1:1000) were conducted overnight in the presence of 0.01% Triton-X100 and 3% normal donkey serum. After washing, the sections were incubated for 1 h at room temperature with AlexaFluor 488- or AlexaFluor 594-conjugated secondary antibodies (Molecular Probes, 1:200), washed and mounted on slides with vectashield (Vector Labs) and images were acquired with a fluorescence microscope.

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***Radioligand binding assays in nerve terminal membranes***

Nerve terminals from the striatum of male R6/2-CAG120 [B6CBA-Tg(HDexon1)62Gpb/3J from Jackson Laboratories] with 35-40 days were purified using combined sucrose and Percoll fractionation, as previously described (Rodrigues et al., 2008). The purity of the nerve terminals was confirmed by immunocytochemistry since over 95% of visualized elements were immunopositive for synaptophysin and less than 5% were immunopositive for GFAP or PSD95, i.e. they mostly corresponded to the presynaptic component of synapses (Rodrigues et al., 2008). The density of $A_{2A}R$ was estimated with a radioligand binding assay using a supra-maximal concentration of the $A_{2A}R$ antagonist [$^3$H]SCH58261 (6 nM; offered by Dr. E.Ongini, Schering-Plough, Italy), as previously described (Lopes et al., 2004). Binding reactions were for 1 h at room temperature (23±1ºC) with 24-36 µg of protein in a final volume of 200 µL of a Tris-Mg solution (50 mM Tris and 2 mM $MgCl_2$ pH 7.4) and 4 U/mL of adenosine deaminase, with constant swirling. Specific binding was determined by subtraction of non-specific binding, measured in the presence of 3 µM XAC (Tocris, Bristol, UK), a mixed $A_1R/A_2R$ antagonist. Binding reactions were stopped by addition of 4 mL of ice-cold Tris-Mg solution and filtration through Whatman GF/C glass microfiber filters (GE Healthcare) in a filtration system (Millipore). The radioactivity was measured at least 12 hours after adding 2 mL of scintillation liquid (AquaSafe 500Plus, Zinsser) in a Tricarb β-counter (Perkin Elmer). All these binding assays were performed in triplicate and the values are presented as mean±S.E.M. of n=3-4 (i.e. in preparation obtained from different mice) and expressed as fmol/mg protein.

***Synaptic plasticity at cortico-striatal synapses***

Electrophysiological recordings of cortico-striatal transmission were carried out essentially as previously described (Quiroz et al., 2009). Coronal cortico-striatal slices (400 µm thick) were prepared from male R6/2-CAG120 [B6CBA-Tg(HDexon1)62Gpb/3J from Jackson Laboratories] with 35-40 days, and allowed to equilibrate for at least 90 min at room temperature in modified artificial cerebrospinal fluid (aCSF) containing (in mM); 124 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26

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$NaHCO_3$, 1.2 $NaH_2PO_4$ and 10 D-glucose, bubbled with a gas mixture of 95% $O_2$ / 5% $CO_2$. One slice was transferred to a submerged recording chamber and continuously superfused with gassed aCSF at a flow rate of 3 mL/min. Stimulation was delivered at a frequency of 0.05 Hz through a bipolar twisted tungsten wire placed in the white matter above the dorsolateral striatum. Extracellular field recordings were obtained with micropipettes (2–4 MΩ) filled with 4 M NaCl, placed in the dorsolateral striatum, to record population spike responses to estimate synaptic efficacy. Recordings were obtained with an ISO-80 amplifier (World Precision Instruments) and digitized using an ADC-42 board (Pico Technologies). Averages of 4 consecutive responses were continuously monitored on a personal computer with the LTP 1.01 software (Anderson and Collingridge, 2001).

Synaptic plasticity was triggered by frequency stimulation trains, applied at least 30 min after obtaining a stable baseline: to trigger long-term potentiation (LTP), we applied three high frequency stimulation trains of 100 Hz for 1 second each delivered every 5 seconds, whereas to trigger long-term depression (LTD), we applied a low frequency train of 10 Hz for 10 min. The amplitude of synaptic plasticity (LTP or LTD) was quantified as the percentage change between two values: the average amplitude of the five potentials taken between 35 and 45 min after the end of the induction protocol in relation to the average amplitude of the field potentials measured during 10 min that preceded the induction protocol.

To test the role of $A_{2A}R$ on synaptic plasticity, we used the selective antagonist SCH58261 {7-(2- phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazol-ol[1,5c]pyrimidine; from Tocris} at a selective but supra-maximal concentration of 50 nM (Lopes et al., 2004). SCH58261 was made up as a 5 mM stock solution in dimethylsulfoxide, and dissolved in aCSF solution to a concentration of 50 nM.

***Statistical Analysis***

Single statistical comparisons between two groups were analyzed using a non-paired two-tailed Student's *t* test. Statistical comparison of behavioral parameters at multiple time points was

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analyzed by repeated measure ANOVA or one-way ANOVA followed by a Tukey's multiple-comparison post hoc test or by a Dunnett's multiple-comparison post hoc test (for comparison with specific controls). Data with more than one variable and/or condition were analyzed with a two-way ANOVA followed by Bonferroni post hoc tests. Survival data were analyzed using the Mantel-cox log-rank test and Kaplan-Meier survival curves. Unless otherwise indicated, the significance level was 95%.

## Results

### *Genetic inactivation of $A_{2A}$R reversed working memory deficit in R6/2 mice*

We focused on working memory, which is defined as the capacity to maintain information held "online" to select appropriate behavioral responses (Goldman-Rakic, 1996), for three reasons: i) it is typically impaired in HD models; ii) it is associated with a dysfunction of the cortico-striatal pathway; iii) it is modulated by the striatal $A_{2A}$R in normal animals. Using an 8-arm radial maze, we found that CAG120-WT mice at postnatal week 6 displayed significant deficits of working memory compared to WT-WT mice (Fig.1A, n=12-15/group). Genetic inactivation of $A_{2A}$R selectively reduced working memory errors in normal (WT-$A_{2A}$R KO compared to WT-WT mice) as well as in CAG120 mice (CAG120-$A_{2A}$R KO compared to CAG120-WT) (Fig. 1A), without significant effect on reference memory (Fig. 1B). When tested in the rotarod test (Fig. 1C, n=13-18/group), we observed that CAG120-WT mice at postnatal week 6 displayed locomotor deficits typified by a shorter latency to fall from the rotating platform, compared to WT-WT mice. Notably, the genetic inactivation of $A_{2A}$R did not modify the rotarod performance in WT mice (WT- $A_{2A}$R KO compared to WT-WT mice) nor did it recover performance in CAG120 mice (CAG120- $A_{2A}$R KO compared to CAG120-WT) (Fig. 1C). Thus genetic inactivation of $A_{2A}$R selectively rescued working memory deficits, independently of locomotor deficits.

To overcome the difficulty in assessing cognition associated with the rapid onset of motor

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deficits in the R6/2-CAG120 mice, we analyzed another R6 mouse line (R6/2-CAG240), which has a longer presymptomatic period with a delayed presentation of motor symptom and is better suited for cognitive behavioral analysis during the prodrome. Thus, CAG240-WT mice (n=12-16/group) did not display locomotor deficits in the rotarod test at postnatal month 2 (Fig. 2C), which became evident at postnatal month 3 (Fig. 2G). However, consistent with cognitive dysfunction being a characteristic of HD prodrome, CAG240-WT mice at postnatal month 2 already displayed deficits of working memory (particularly on the testing day 3; Fig. 2A) and of short-term reference memory (on the testing day 2-3; Fig. 2B), compared to WT littermates (WT-WT). These cognitive deficits were maintained upon emergence of motor deficits, since CAG240-WT mice at postnatal month 3 still displayed clear deficits of working memory (testing days 4-5; Fig. 2D) and reference memory (testing days 3-4; Fig. 2E). Importantly, the genetic inactivation of $A_{2A}R$ selectively reversed working and reference memory deficits (comparing CAG240-$A_{2A}R$ KO with CAG240-WT mice) both at postnatal month 2 (Fig. 2A, B) and at postnatal month 3 (Fig. 2D, E), without effects on locomotion tested in the rota-rod test (Fig. 2C, F).

Together, these findings show for the first time that the genetic inactivation of $A_{2A}R$ reverses the impairment of working memory in R6/2 mice of both CAG120 and CGA240 lines, independently of locomotor modifications.

***Pharmacological blockade of $A_{2A}R$ also reversed working memory deficit in R6/2 mice***

We next investigated if the pharmacological blockade of $A_{2A}R$ mimicked the beneficial impact of the genetic $A_{2A}R$ inactivation on the working memory deficits of R6/2 mice. As shown in Figure 3A, the treatment of R6/2-CAG240 mice at postnatal month 3 with the selective $A_{2A}R$ antagonist KW6002 (1 mg/kg) significantly ameliorated working memory performance (n=7-8). Notably, this dose of KW6002, which produces little effect on motor activity (as indicated by their entrance frequencies to the arm, Fig. 3C), did not affect reference memory in R6/2-CAG240 mice at postnatal month 3 (Fig. 3B).

***Blockade of $A_{2A}R$ did not modify ubiquitin-positive neuronal inclusions, astrogliosis or***

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***DARPP-32 activity in R6/2 mice.***

To foster the mechanism underlying the ability of $A_{2A}R$ blockade to rescue working memory deficits in HD, we carried out a qualitative screening to examine the effect of genetic $A_{2A}R$ inactivation on some candidate mechanisms currently explored to manage HD, namely the ubiquitin-proteasome system (Seo et al., 2004), the deposition of the mutant huntingtin protein (DiFiglia et al., 1997), reactive astrogliosis (Bradford et al., 2009; Selkoe et al., 1982) and aberrant cortico-striatal transmission (Cepeda et al., 2007; Hohn et al., 2011; Hong et al., 2012).

As shown in Figure 4A, R6/2-CAG120 mice at postnatal day (PN) 70 (a relatively late stage of this line) displayed a scatter distribution pattern of ubiquitin-positive neuronal intranuclear inclusions in some areas of the cortex and striatum, which were indistinguishable between CAG120-WT and CAG120-$A_{2A}R$ KO mice. We also examined R6/2-CAG240 mice at postnatal month 2, which did not present any ubiquitin-positive neuronal intranuclear inclusions in the cortex or striatum regardless of $A_{2A}R$ genotype (data not shown).

We next used GFAP immunoreactivity as an index of reactive astrogliosis (Pekny and Nilsson, 2005). R6/2-CAG240 mice at postnatal month 3 displayed increased GFAP staining in the white matter between cortical and striatal regions and in the cortex (insert image from the box location in Fig. 4B), which was indistinguishable between CAG240-WT and CAG240-$A_{2A}R$ KO mice (Fig. 4B). A similar GFAP staining was observed in CAG120 mice at PN50 and, again, there was no significant difference of GFAP immunoreactivity between R6/2-WT and R6/2-$A_{2A}R$ KO (Fig. 4B).

To obtain a general estimate of a possible influence of the genetic deletion of $A_{2A}R$ on cortico-striatal activity, we compared the immunoreactivity of DARPP-32 (dopamine- and cAMP–regulated phosphoprotein) phosphorylated at threonine-75, which has been proposed as marker of the glutamatergic drive of medium spiny neurons (Nishi et al., 2005) that we previously showed to be modulated by presynaptic $A_{2A}R$ (Shen et al., 2013). As shown in Figure 4C, immunoreactivity for DARPP-32 phosphorylation at Thr-75 (32-Thr-75-p) was largely enriched in striatum.

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Importantly, DARPP-32-Thr-75-p levels were markedly reduced in R6/2-WT and WT-$A_{2A}$R KO compared to WT-WT. Double transgenic mice (i.e. R6/2-$A_{2A}$R KO) did not show any further reduction in DARPP-32-Thr-75-p compared to WT-$A_{2A}$R KO or R6/2-WT alone (CAG120 line at PN50).

***Increased density and gain of function of $A_{2A}$R to control of long-term depression in R6/2 mice***

We detected an increase of the binding density of $^{3}$H-SCH58261 (6 nM) in nerve terminal membranes of R6/2 mice at postnatal week 6 (953±51 fmol/mg protein, n=5) compared to WT mice (695±8 fmol/mg protein, n=5) (Fig. 4D). This prompted investigating a possible gain of function of $A_{2A}$R in the control of synaptic plasticity in cortico-striatal synapses, which is the most relevant neurophysiological role of $A_{2A}$R (Cunha, 2008) and is critically involved in reverting abnormal changes of synaptic plasticity and behavior (Batalha et al., 2013). In *ex vivo* slice electrophysiological experiments, we found that the amplitude of low frequency-induced long-term depression (LTD) in cortico-striatal synapses was similar ($p<0.05$) in WT and R6/2 mice (Fig. 5A, B), without changes in the input-ouput curves between the two groups of mice (data not shown). However, whereas the blockade of $A_{2A}$R with the selective antagonist SCH58261 (50 nM) did not significantly affect LTD amplitude in WT mice (Fig. 5A, C), $A_{2A}$R blockade significantly ($p<0.05$) decreased LTD amplitude in slices from R6/2 mice (Fig. 5A, D).

Interestingly, there was a selective modification of the impact of $A_{2A}$R in the control of LTD rather than in the control of high frequency-induced long-term potentiation (LTP) at cortico-striatal synapses (Fig. 5E-H). In fact, the amplitude of LTP was similar ($p>0.05$) between WT and R6/2 mice (Fig. 5E, F) and SCH58261 (50 nM) decreased LTP ($p<0.05$) in WT (Fig. 5E, G) and R6/2 mice (Fig. 5E, H) to a similar extent (Fig. 5E; $p>0.05$). This impact of $A_{2A}$R on synaptic plasticity contrasted with the lack of effect of SCH58261 on basal synaptic transmission (data not shown), confirming that $A_{2A}$R are also selectively engaged to control synaptic plasticity phenomena (Cunha, 2008) at cortico-striatal synapses.

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***Long-term impact of the genetic $A_{2A}R$ inactivation on the survival of R6/2 mice***

In order to bolster the therapeutic interest of blocking $A_{2A}R$ to manage cognitive impairments in early HD, we next investigated if the genetic inactivation of $A_{2A}R$ caused long-term detrimental effects in R6/2 mice by investigating their survival. We report that the genetic inactivation of $A_{2A}R$ in CAG120 mice markedly increased their survival (PN134) compared to the $A_{2A}R$ WT background (PN85) (Fig 6A). Furthermore, CAG120-$A_{2A}R$ heterozygous KO mice showed intermediate improvement (average PN99), suggesting a specific $A_{2A}R$ gene dose effect on survival. Intriguingly, we noted that this protective phenotype in R6/2-CAG120-$A_{2A}R$ KO mice appeared to be associated with an attenuated seizure activity (gauged by visual inspection), well characterized in this mouse line upon aging (Mangiarini et al., 2006): CAG120-WT developed seizure activity more frequently than CAG120-$A_{2A}R$ KO, a phenotype that might be related to the mixed genetic background (*C57BL6J x CBAJ)*. Although this contention was based on a non-quantitative analysis, it is re-enforced by the lack of effect of $A_{2A}R$ inactivation in R6/2-CAG240 (Fig. 6B), generated in congenic C57BL6 background and which developed significantly less seizure activity (Mangiarini et al., 2006).

## Discussion

By combining the use of double transgenic with $A_{2A}R$ deletion from two transgenic lines (R6/2- CAG120 and R6/2-CAG240) modeling Huntington's disease (HD), with the selective $A_{2A}R$ antagonist KW6002, we show here that $A_{2A}R$ blockade reverts the deficits of working memory both at prodrome and early phases of HD R6/2 mice, recovering the performance of control mice. We also found an up-regulation of synaptic $A_{2A}R$ in early HD and a gain of function of $A_{2A}R$ to control LTD in cortico-striatal synapses, which are aberrantly altered in early HD (*e.g.* Cepeda et al., 2007; Höhn et al., 2011; Hong et al., 2012) and $A_{2A}R$ antagonists reverse it. These findings prompt the exciting possibility that striatal $A_{2A}R$ may represent a novel target against the cognitive inflexibility (such as working memory impairment) in the HD prodrome (Papp et al., 2011; Stout et al., 2011).

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The conclusion that $A_{2A}R$ rescue the deficits of working memory in early HD extends to a pathological condition the striking ability of $A_{2A}R$ to control working memory and cognitive flexibility (Augusto et al., 2013; Wei et al., 2011; Yu et al., 2009; Zhou et al., 2009). This beneficial impact resulting from $A_{2A}R$ deletion on working memory had a qualitatively similar impact in the two R6/2 mouse lines used in the present study, which have a different genetic background, thus increasing the robustness of our present conclusion. The use of transgenic mice with cell type-selective deletions of $A_{2A}R$ has previously enabled us to determine that it was the specific population of $A_{2A}R$ in cortico-striatal pathways that was responsible for the control of working memory and habit formation in normal animals (Augusto et al., 2013; Wei et al., 2011; Yu et al., 2009; Zhou et al., 2009). Remarkably, several studies have shown that it is precisely cortico-striatal pathways that undergo the most precocious neurophysiological alterations that parallel cognitive impairment in the prodrome of HD (Cepeda et al., 2007; Hong et al., 2012; Joshi et al., 2009; Klapstein et al., 2001). Indeed, before the emergence of motor deficits, there is a parallel hyper-excitability (Cepeda et al., 2007; Joshi et al., 2009; Klapstein et al., 2001) and partial disconnection of the cortico-striatal pathway (Hohn et al., 2011; Hong et al., 2012) that is associated with alterations of synaptic plasticity (Dalbem et al., 2005; Kung et al., 2007). This imbalance of synaptic plasticity has been proposed to be the neurophysiological basis of striatal-dependent learning (reviewed in Lovinger et al., 2010), namely the alterations of long-term depression (LTD) (Kheirbek et al., 2009; Lovinger, 2010). We now observed that $A_{2A}R$ selectively controlled LTD without affecting basal synaptic transmission or the glutamate-dependent phosphorylation of threonine-75 of DARPP-32 in the targeted medium spiny neurons. This simultaneous selective control of LTD in cortico-striatal pathways and working memory dysfunction in R6/2 mice upon genetic or pharmacological blockade of $A_{2A}R$, thus suggests that the gain of function of $A_{2A}R$ to control striatal LTD may underlie the ability of $A_{2A}R$ blockade to revert working memory deficits in R6/2 mice, an hypothesis that still needs additional experimental confirmation. It also remains to be determined if this ability of $A_{2A}R$ to control striatal LTD and working memory dysfunction may involve the ability of $A_{2A}R$ to modulate either NMDA receptors (Rebola et al., 2008; Tebano et al., 2005; Wirkner et al., 2004) that play a key role to induce striatal synaptic plasticity (e.g. Lovinger, 2010), or BDNF

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bioavailability (Minghetti et al., 2007) and its impact on the expression of synaptic plasticity (reviewed in Sebastião and Ribeiro, 2009), or cannabinoid CB1 receptors (Ferré et al., 2010; Martire et al., 2011) also involved in LTD expression (Gerdeman et al., 2002), or dopamine D2 receptor function (Tozzi et al., 2007) to shift the balance of synaptic plasticity in the striatum (Shen et al., 2008). Furthermore, our present conclusion that the $A_{2A}R$-mediated control of cortico-striatal plasticity underlies the impact of $A_{2A}R$ on working memory dysfunction does not exclude the possibility that there might be an additional participation of $A_{2A}R$ controlling neuronal circuits in other brain regions (Bannon et al., 2014; Rebola et al., 2008) where abnormal information flow has also been found in association with cognitive impairments in early HD, such as the hippocampus (e.g. Milnerwood et al., 2006; Milnerwood and Raymond, 2010), prefrontal (Dallerac et al., 2011) or perirhinal cortex (Cummings et al., 2006).

This gain of function of $A_{2A}R$ to control striatal LTD and working memory deficits in R6/2 mice was associated with an up-regulation of $A_{2A}R$ in the presynaptic compartments in the striatum of R6/2 mice at motor presymptomatic phases, as also observed by others to occur very transiently at early time points, although using total striatal membranes (Tarditi et al., 2006). A similar up-regulation of $A_{2A}R$ was observed in the afflicted brain regions in different neurodegenerative diseases such as Alzheimer's disease (Albasanz et al., 2008; Espinosa et al., 2013), early life convulsions (Cognato et al., 2010) or attention deficit and hyper-activity disorder (Pandolfo et al., 2013), as well as upon aging (Canas et al., 2009; Rebola et al., 2003), all conditions where $A_{2A}R$ blockade prevents spatial memory impairment (Canas et al., 2009; Cognato et al., 2010; Dall'igna et al., 2007; Prediger et al., 2005). Notably the up-regulation of $A_{2A}R$ upon noxious brain insults is most evident in glutamatergic synapses (Cognato et al., 2010; Duarte et al., 2012; Rebola et al., 2005b), in accordance with the suggestion that $A_{2A}R$ blockade controls learning disabilities through a normalization of aberrant plasticity in excitatory synapses (reviewed in Cunha and Agostinho, 2010). This is further supported by the lack of modification of other processes that have been also associated with early dysfunction in HD and that can be controlled by $A_{2A}R$; thus, our qualitative screening indicated that the genetic inactivation of $A_{2A}R$ in R6/2 mice did not modify either the

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deposition of huntingtin and the emergence of ubiquitin-containing nuclear inclusions that are hallmarks of HD (*e.g*. DiFiglia et al., 1997; Seo et al., 2004) and can be controlled by $A_{2A}R$ (Chiang et al., 2009; Huang et al., 2011) or the putative astrogliosis, measured as an increased GFAP immunoreactivity that is also part of HD neuropathology (Bradford et al., 2009; Selkoe et al., 1982) and can be controlled by $A_{2A}R$ (Matos et al., 2012; Minghetti et al., 2007). Although the observed up-regulation of presynaptic $A_{2A}R$, their gain of function in the control of cortico-striatal LTD and the selective amelioration of working memory deficits in R6/2 (but not WT) mice by KW6002, is suggestive of a selective ability of $A_{2A}R$ to control the working memory deficits associated with the over-expression of mutated huntingtin, the present results do not actually allow disentangling this proposal from the equally likely possibility that the amelioration of working memory performance upon $A_{2A}R$ deletion might instead result from an additive effect of the detrimental impact of huntingtin mutation and of the beneficial effect of $A_{2A}R$ deletion on working memory performance also observed in control animals (Augusto et al., 2013; Wei et al., 2011; Yu et al., 2009; Zhou et al., 2009).

This selective benefit upon $A_{2A}R$ blockade to rescue working memory deficits in prodrome and early HD should be clearly distinguished from the reported effects of $A_{2A}R$ in the locomotor alterations that are characteristic of middle and late stages on HD. Several studies have reported dual and opposite effects of $A_{2A}R$ on HD-associated neurochemical and motor disturbances during the evolution of HD (reviewed in Popoli et al., 2007), so that *$A_{2A}R$ blockade* seems beneficial at *early* stages whereas it is *$A_{2A}R$ activation* that becomes beneficial at *later* stages of the disease (Chiang et al., 2009; Chou et al., 2005; Domenici et al., 2007). Notably, this is closely correlated with electrophysiological and neurochemical evidence suggesting that the cortico-striatal glutamatergic pathway undergoes a biphasic change, from hyperactivity in the early, pre-symptomatic disease stage, to depression at late, symptomatic stages of HD models (Cepeda et al., 2007; Hong et al., 2012; Joshi et al., 2009; Klapstein et al., 2001). This modification of the overall impact of $A_{2A}R$ blockade during the course of the disease is also mirrored by the previously reported transient nature of the up-regulation of striatal $A_{2A}R$ protein and mRNA levels during the

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course of the disease (Tarditi et al., 2006). This further argues for a critical role of synaptic $A_{2A}R$ controlling cortico-striatal plasticity in the recovery of working memory selectively in the prodrome and early HD, independently of the impact of $A_{2A}R$ on motor function or neuronal death at later stages of HD. This contention is also in agreement with the long-standing view that HD begins with a synaptic dysfunction (synaptopathy) preceding neuronal death (Deng et al., 2013; Li et al., 2003; Milnerwood and Raymond, 2010), which selectively affects corticostriatal projections (Cepeda et al., 2007; Hong et al., 2012; Joshi et al., 2009; Klapstein et al., 2001). Additionally, it should be emphasized that the presently demonstrated impact of $A_{2A}R$ on working memory deficits are pertinent to the prodrome of HD and likely attributed to the presynaptic $A_{2A}R$ function, and should be distinguished from the lack of direct effect of $A_{2A}R$ alone, unless dopamine $D_1$ receptors are also simultaneously targeted, to control long-term reference memory deficits that emerge at later stages of HD and largely derived from the postsynaptic $A_{2A}R$ (Tyebji et al., 2014).

The therapeutic interest of $A_{2A}R$ antagonists to control cognitive impairments in early HD is further bolstered by our findings that the inactivation of $A_{2A}R$ in R6/2 mice does not seem to have long-term deleterious consequences as gauged by the lack of a decrease of the survival of R6/2 mice. In fact, $A_{2A}R$ have been proposed to be associated with the onset of motor symptoms (Dalbem et al., 2005) and with survival (Mievis et al., 2011) in HD patients and animal models. We now observed that $A_{2A}R$ inactivation increased the survival of R6/2-CAG120 mice, while it did not significantly affect the survival of R6/2-CAG240 mice. This probably results from the ability of $A_{2A}R$ to control seizure activity (El Yacoubi et al., 2008; El Yacoubi et al., 2009) limiting the survival of R6/2-CAG120 mice due to their mixed genetic background, a feature that is eliminated in R6/2-CAG240 mice that have a in congenic C57BL/6 background. However, this revealed therapeutic interest of $A_{2A}R$ antagonists to manage the working memory dysfunction characteristic of the HD prodrome should be carefully balanced with the current lack of consensus about the therapeutic interest of $A_{2A}R$ in the control of motor abnormalities characteristic of HD (Popoli et al., 2007); in fact, several studies have failed to document a motor benefit or increased survival resulting from caffeine consumption in HD patients (Simonin et al., 2013) or $A_{2A}R$ antagonism in animal models of

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HD (Mievis et al., 2011) and instead concluded on the motor benefits of $A_{2A}R$ agonists to limit the abnormal motor phenotype of overt HD (Chou et al., 2005). This means that the window of interest to antagonize $A_{2A}R$ to manipulate working memory deficits might be limited to the prodrome phase of HD.

In conclusion, the present study shows the ability of $A_{2A}R$ inactivation or pharmacological blockade to control working memory deficits in the prodrome and early phases of R6/2 mice modeling HD. This was associated with an up-regulation of synaptic $A_{2A}R$ and a gain of function of $A_{2A}R$ to selectively control LTD in cortico-striatal pathways. Together with the lack of deleterious long-term consequences, these findings provide a proof-of-concept to propose $A_{2A}R$ antagonists as a new therapeutic option to manage cognitive impairments and improve the quality of life of patients in the prodrome and early HD, a proposal that still needs to be validated by clinical trials that can be rapidly implemented since $A_{2A}R$ antagonists have now been approved as anti-Parkinsonian drugs with a notable safety profile.

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**Figure legends**

**Figure 1. Genetic inactivation of $A_{2A}R$ selectively rescues working memory deficits, independently of locomotor deficits at post-natal week 6 in the R6/2 (CAG120) mice.**

Double transgenic CAG120-$A_{2A}R$ KO and their WT littermates were generated and tested for working memory at postnatal week 6 as described in the Methods. CAG120-WT mice displayed significant deficits of working memory compared to WT-WT mice. Genetic inactivation of $A_{2A}R$ preferentially prevented working memory error in normal as well as in CAG120 mice (**A**), without significant effect on reference memory (**B**). CAG120-WT mice at postnatal week 6 displayed locomotor deficits, compared to WT-WT mice (**C**), but the genetic inactivation of $A_{2A}R$ did not modify the rota-rod performance in WT mice nor did it recover performance in CAG120 mice (**C**). Repeated measure ANOVA or one-way ANOVA followed by a Tukey's multiple-comparison post hoc test. * $P<0.01$, #$P<0.05$, n=12-15 for the 8-arm maze test; n=13-18 for the rotarod test.

**Figure 2. Genetic inactivation of $A_{2A}R$ prevents the impairment of working memory in R6/2 (CGA240) mice, independently of locomotor modifications.**

Double transgenic CAG120-$A_{2A}R$ KO and their WT littermates were generated and tested for working memory at postnatal month 2 and 3 as described in the Methods. At postnatal month 2, CAG240-WT mice displayed no motor deficits (**C**), but a clear deficits of working memory (**A**) and short-term reference memory (on testing days 2-3; **B**), compared to WT littermates (WT-WT). These cognitive deficits were maintained upon emergence of motor deficits at postnatal month 3 (testing days 4-5; **D**) and reference memory (testing days 3-4; **E**). Importantly, the genetic inactivation of $A_{2A}R$ selectively reversed working and short-term reference memory deficits (comparing CAG240-$A_{2A}R$ KO with CAG240-WT mice) both at postnatal month 2 (**A, B**) and postnatal month 3 (**D, E**), without effects on locomotion in the rota-rod test (**C, F**). Repeated measure ANOVA or one-way ANOVA followed by a Tukey's multiple-comparison post hoc test. *P<0.01, #P<0.05, n=12-16.

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**Figure 3. Pharmacological blockade of $A_{2A}R$ also reverses working memory deficits in R6/2 (CGA240) mice at postnatal month 3.**

R6/2-CAG240 mice at postnatal month 3 were treated with the $A_{2A}R$ antagonist KW6002 (1 mg/kg, i.p.) and tested 30 min after for their working and reference memory using the 8 arm radial maze. KW6002 significantly ameliorated working memory performance (**A**) without effects on short-term reference memory (**B**) and locomotor activity (as indicated by the entrance frequency to the arm (entrances/10 sec) (**C**) in R6/2-CAG240 mice. Repeated measure ANOVA followed by a Tukey's multiple-comparison post hoc test. *P<0.01, #P<0.05, n=7- 8.

**Figure 4. Genetic inactivation of $A_{2A}R$ does not affect ubiquitin-positive neuronal inclusion, astrogliosis and DARPP-32 phosphorylation in R6/2 mice.**

Double transgenic CAG120-$A_{2A}R$ KO, CAG240-$A_{2A}R$ KO and their WT littermates were sacrificed for immunohistochemical analysis of ubiquitin (**A**), astrogliosis (**B**) and DARPP-32 phosphorylation at threonine-75 (DARPP-32-Thr-75, **C**), as described in the Methods. (**A**) At postnatal weeks 10, there was no qualitative difference in ubiquitin-positive neuronal intranuclear inclusions in either the cerebral cortex or striatum between CAG120-$A_{2A}R$ KO and CAG120-WT mice. Red color-ubiquitin positive; Blue color-DAPI positive. (**B**) At postnatal day (PN) 50, R6/2-CAG120 mice displayed increased GFAP-immunoreactivity, particularly in the white matter between cortical and striatal regions and in the cortex (insert image from the box location in Fig. 4B). However, the pattern of GFAP-immunoreactivity was indistinguishable between CAG120-WT and CAG120-$A_{2A}R$ KO mice (upper panels). Similarly, GFAP immunoreactivity was indistinguishable between CAG240-WT and CAG240-$A_{2A}R$ KO at PN 90 (lower panels). (**C**) At PN 50, the immunoreactivity of DARPP-32-Thr-75 was markedly reduced in CAG120-$A_{2A}R$ KO and CAG120-WT mice compared with WT-WT littermates; however, double transgenic mice (i.e. CAG120-$A_{2A}R$ KO) did not show any further modification of DARPP-32-Thr-75-p compared to WT-$A_{2A}R$ KO or R6/2-WT alone (40x). (**D**) The specific binding density of $^{3}$H-SCH58261 in R6/2 mice and WT littermates at PN45, as described in the Methods, showed that the binding density of $^{3}$H-SCH58261 in nerve terminal membranes of R6/2 mice was larger compared to their WT littermates

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(*P<0.05, unpaired Student's *t* test, n=5).

**Figure 5. Effect of $A_{2A}$R on LTD and LTP in cortico-striatal synapses of R6/2 mice.**

Different patterns of high frequency stimulation (HFS) of the afferent cortical projections triggered LTP and LTD in the dorso-lateral striatum evaluated as changes of the amplitude of population spike (PS) electrophysiological recordings in slices collected from R6/2 mice and WT littermates at postnatal day 35, as described in the Methods. The amplitude of LTD in cortico-striatal synapses was similar (p<0.05) in WT and R6/2 mice (**A, B**); however, the blockade of $A_{2A}$R by SCH58261 (50 nM) decreased LTD amplitude only in slices from R6/2 mice (**A, D**), and was devoid of effects in WT mice (**A, C**). The amplitude of LTP was also similar between WT and R6/2 mice (**E, F**) and SCH58261 (50 nM) decreased LTP in WT (**E, G**) and R6/2 mice (**E, H**) to a similar extent (**E**). (*P<0.05, unpaired Student's *t* test, n=4-5).

**Figure 6. Genetic inactivation of the $A_{2A}$R improved survival of CAG120 but not CAG240 mice.**

Double transgenic CAG120-$A_{2A}$R homozygous (KO) and heterozygous (HE) and their WT littermates were generated and monitored for survival during postnatal development as described in the Methods. The survival data were analyzed by Kaplan-Meier survival curves analysis. (**A**) Genetic inactivation of $A_{2A}$R improved the survival time of the CAG120 line. CAG120-$A_{2A}$R-WT, n=8; CAG120-$A_{2A}$R-KO, n=7; CAG120-$A_{2A}$R-HE, n=10. (**B**) Survival of the CAG240 line. CAG240-$A_{2A}$R WT, n=7; CAG240-$A_{2A}$R-KO, n=8; CAG240-$A_{2A}$R-HE, n=9. A Log-rank (Mantel-Cox) test showed significant difference in CAG120 line ($p$=0.0014) but not in CAG240 line ($p$=0.1648).

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Fig.1

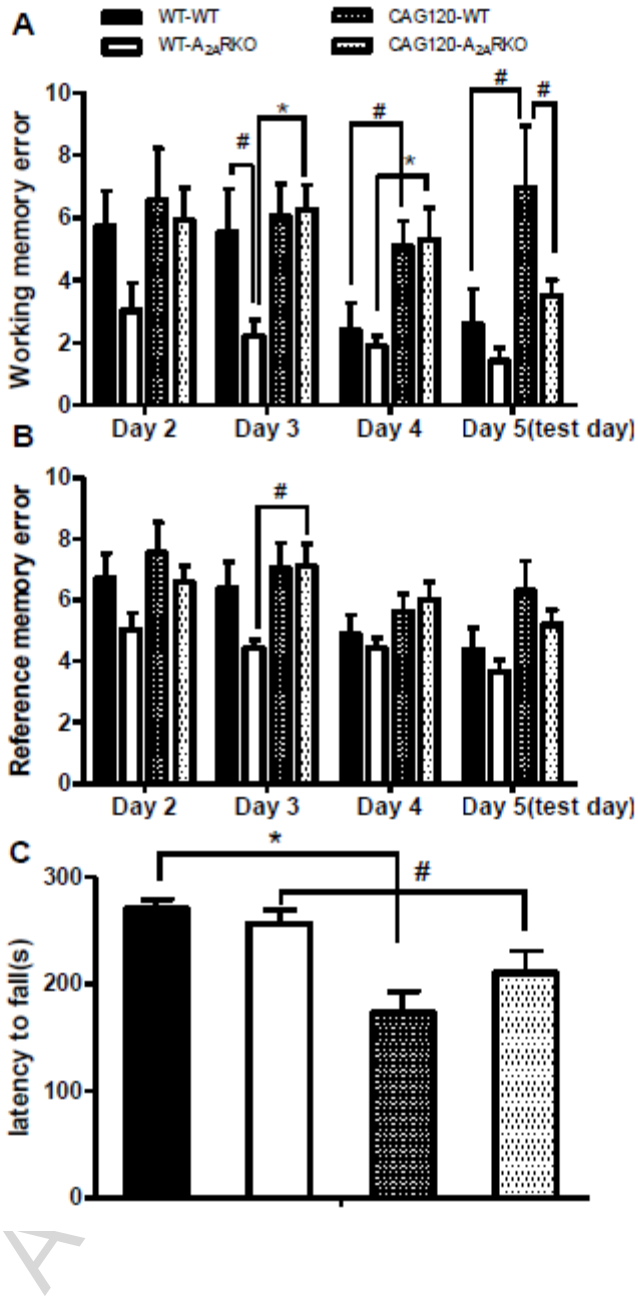

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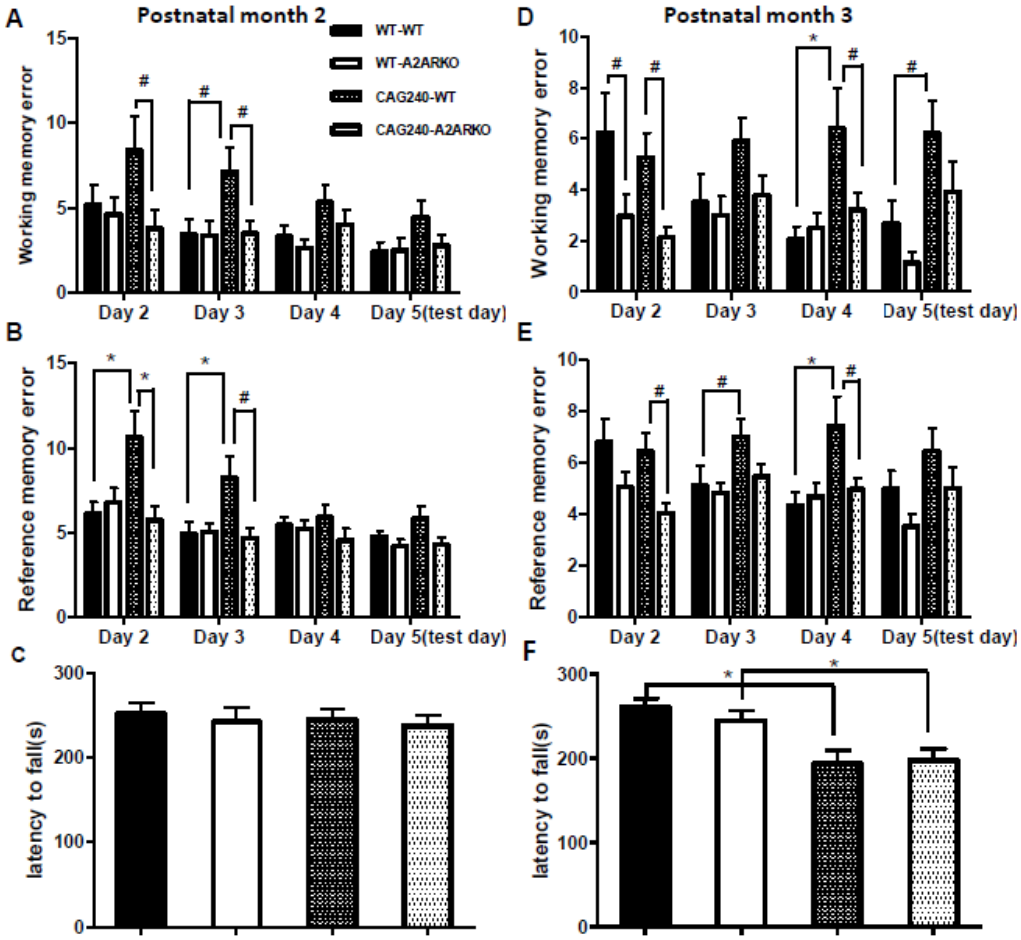
Fig.2

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Li.et al. Figure 3

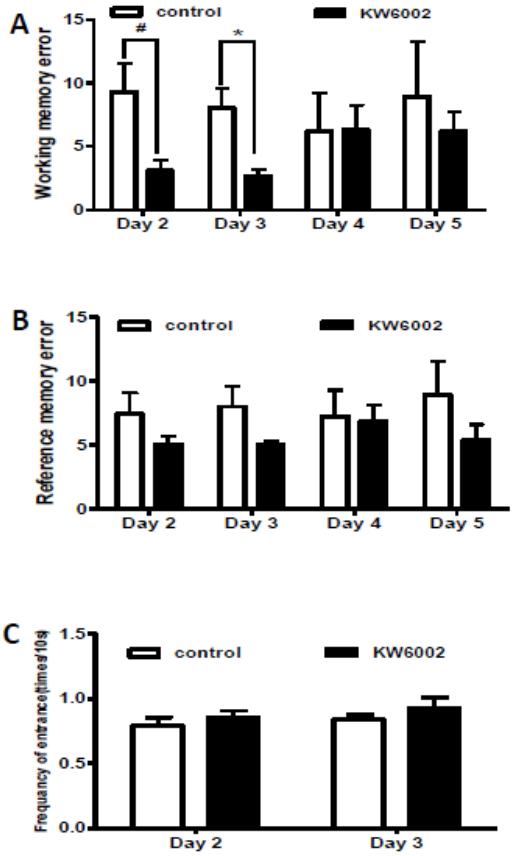


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Figure 4

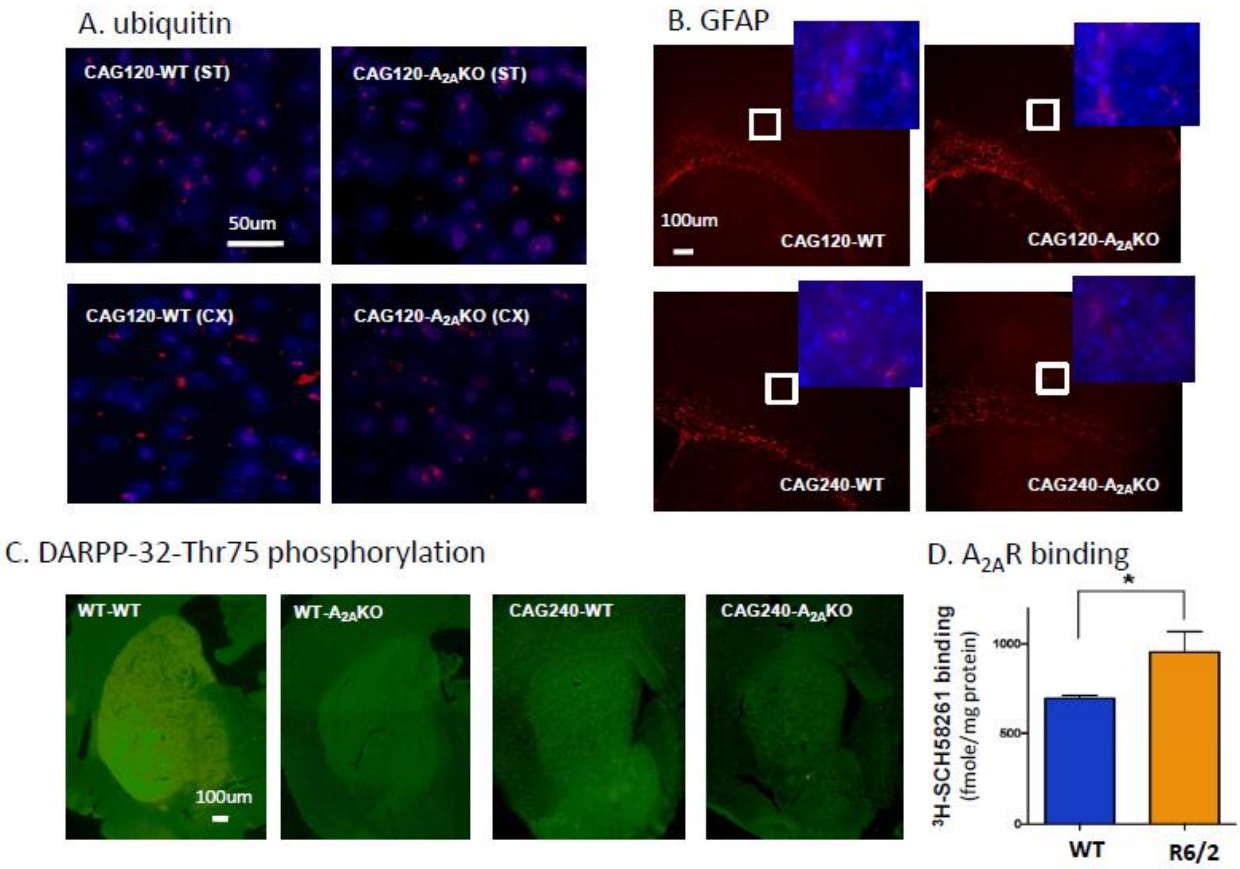


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Figure 5

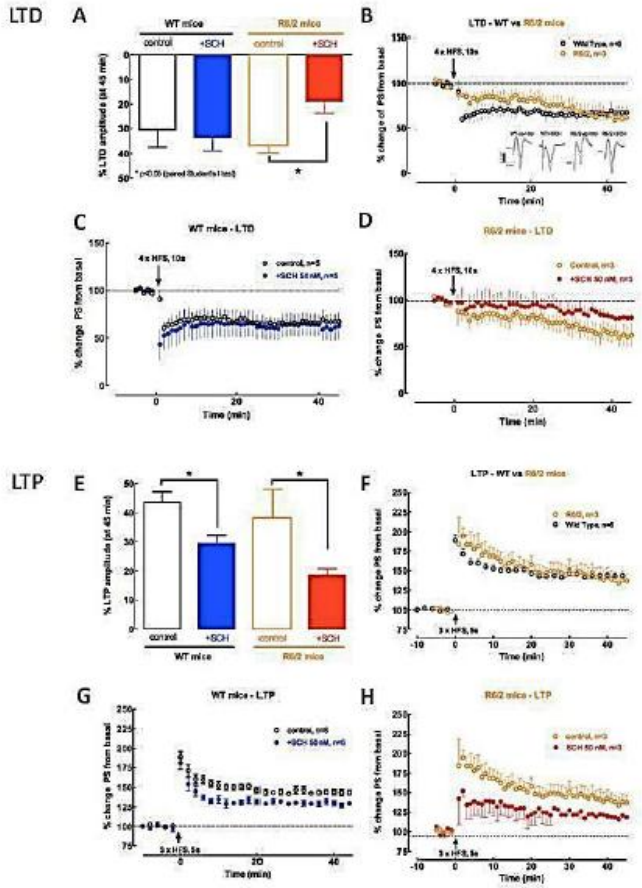


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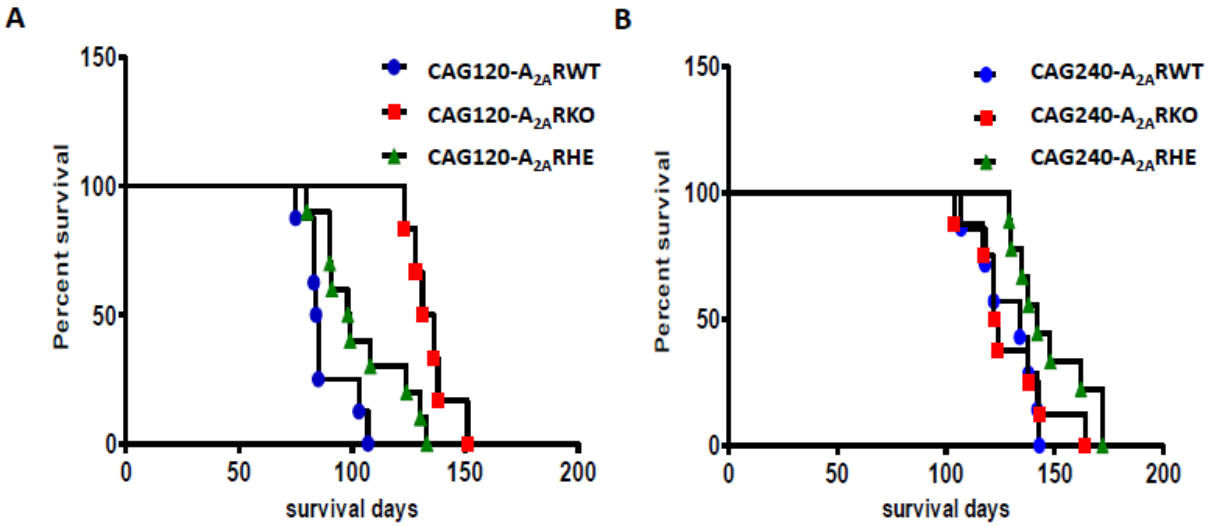
Figure 6

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**Highlight:**

We evaluated the A2A receptor effect on cognitive impairments in two R6/2 lines.

Genetic deletion of A2A receptors reduced working memory deficits in two R6/2 lines

A2A receptor antagonists also reversed working memory deficits in R6/2 mice

A2AR receptor reversed long-term depression abnormality in R6/2 mice

A2A receptor affected cognition in the absence of neurodegeneration in the striatum

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