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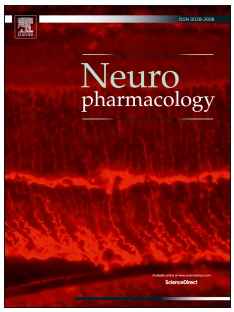

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Adenosine receptors as markers of brain iron deficiency: Implications for Restless Legs Syndrome

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Abstract

Deficits of sensorimotor integration with periodic limb movements during sleep (PLMS) and hyperarousal and sleep disturbances in Restless Legs Syndrome (RLS) constitute two pathophysiologically distinct but interrelated clinical phenomena, which seem to depend mostly on alterations in dopaminergic and glutamatergic neurotransmission, respectively. Brain iron deficiency is considered as a main pathogenetic mechanism in RLS. Rodents with brain iron deficiency represent a valuable pathophysiological model of RLS, although they do not display motor disturbances. Nevertheless, they develop the main neurochemical dopaminergic changes found in RLS, such as decrease in striatal dopamine D_2 receptor density. On the other hand, brain iron deficient mice exhibit the characteristic pattern of hyperarousal in RLS, providing a tool to find the link between brain iron deficiency and sleep disturbances in RLS. The present study provides evidence for a role of the endogenous sleep-promoting factor adenosine. Three different experimental preparations, long-term (22 weeks) severe or moderate iron-deficient (ID) diets (3- or 7-ppm iron diet) in mice and short-term (3 weeks) severe ID diet (3-ppm iron diet) in rats, demonstrated a significant downregulation (Western blotting in mouse and radioligand binding saturation experiments in rat brain tissue) of adenosine A_1 receptors (A1R) in the cortex and striatum, concomitant to striatal D2R downregulation. On the other hand, the previously reported upregulation of adenosine A_{2A} receptors (A2AR) was only observed with severe ID in both mice and rats. The results suggest a key role for A1R downregulation in the PLMS and hyperarousal in RLS.

Keywords

Restless Legs Syndrome; brain iron deficiency; adenosine A_1 receptor; adenosine A_{2A} receptor; dopamine D_2 receptor

1. Introduction

Restless Legs Syndrome (RLS), also known as Willis-Ekbom Disease, is a common neurologic disorder. In the RLS Epidemiology, Symptoms and Treatment (REST) study, 7.2% of US and European adults reported having experienced symptoms of RLS at some point during a 1-year period and 5% reported experiencing symptoms on a weekly basis (Allen et al., 2005). RLS is characterized by a rest-induced, movement-responsive, mostly nocturnal, urge to move the legs commonly associated with periodic leg movements during sleep (PLMS). Sleep disruption is the primary factor producing most of the morbidity (Kushida et al., 2004), but RLS patients do not report sleepiness during daytime despite total sleep times averaging less than 5.5 hours (Allen et al., 2010).

RLS can in fact be pathophysiologically separated into two interrelated clinical phenomena: deficits of sensorimotor integration (PLMS) and hyperarousal (Allen et al., 2010; Ferri et al., 2014, 2015). The interrelation can be demonstrated in polysomnographic studies. Despite the fact that arousals are commonly believed to be caused by PLMS, their onset precedes the onset of PLMS in more than 40% of cases (Ferri et al., 2015). The tight time relationship between PLMS and arousals and their correlated durations seem to indicate that both events might be regulated by an additional mechanism, rather than being connected by a simple reciprocal cause/effect relationship.

Altered dopaminergic function plays an important role in PLMS symptomatology of RLS, which is supported in large part by the remarkable

therapeutic response to L-dopa and dopamine receptor agonists, but also by the repeated demonstration of biochemical changes related to the dopaminergic system (Earley et al., 2014). The dopaminergic profile in RLS particularly includes a decreased density of striatal dopamine D_2 receptors (D2R) and increased tyrosine hydroxylase activity (Connor et al., 2009). On the other hand, glutamatergic mechanisms seem to play a role in the hyperarousal component of RLS. This is supported by the efficacy of ligands of the $\alpha_2\delta$ subunits of the calcium channels (gabapentin and pregabalin) for the sleep disturbances in RLS (Silber et al., 2013; Garcia-Borreguero et al., 2014). Thus, $\alpha_2\delta$-containing calcium channels are mostly localized in glutamatergic terminals (Dooley et al., 2007). The hyperglutamatergic hypothesis is also supported by a recent imaging study using magnetic resonance spectrometry (Allen et al., 2013). In summary, RLS pathophysiology seems to involve two different but interrelated neurotransmitter systems, dopamine and glutamate, each primarily related to different clinical features of RLS. While the dopaminergic system would be mostly related to the deficits in sensorimotor integration (PLMS), glutamate would be mostly involved in hyperarousal and sleep disturbances.

Brain iron deficiency is now well recognized as a main initial pathogenetic mechanism in the development of RLS. The role of brain iron deficiency is based partly on extensive research studies using cerebrospinal fluid, autopsy material, and brain imaging (Rizzo et al., 2013; Earley et al., 2014), indicating reduced regional brain iron, and it is promoting the use of iron therapy in refractory RLS (Silber et al., 2013). Animal models have allowed establishing a causal relation between low

brain iron and altered dopaminergic function in RLS. Brain iron deficiency during the postweaning period in rats or mice produces changes in the dopaminergic system that parallel those found in RLS, mainly the reduced striatal D2R density and an increased mesencephalic TH activity (Connor et al., 2009). Therefore, although rodents with brain iron deficiency do not show motor disturbances, it still represents a valuable pathophysiological model of the PMLS in RLS (Connor et al., 2009). On the other hand, mice with brain iron deficiency show an increase in wakefulness during a particular circadian time point that corresponds to the period during which RLS symptoms are associated with maximal disruption of sleep: during wakefulness preceeding sleep and at the time of sleep onset period (Dean et al., 2006; Ferri et al., 2014).

Therefore, the brain iron deficiency rodent model constitutes a powerful tool to find the link between brain iron deficiency and the alterations in glutamatergic function or other neurotransmitter systems that lead to sleep disturbances in RLS. We present evidence suggesting that alterations in the adenosine system can provide such a link. First, adenosine is a main endogenous sleep-promoting factor. Second, adenosine is a powerful modulator of both glutamate and dopamine neurotransmission (Sebastiao and Ribeiro, 2009; Ferré, 2010; Hines and Haydon, 2014; Pedata et al., 2016). Third, adenosine can also be involved in the motor symptoms of RLS, maybe also providing the link for the interrelation between hyperarousal and PLMS, between alterations in dopamine and glutamate mechanisms.

There are two main subtypes of adenosine receptors in the brain, A_1 and A_{2A}

receptors (A1R and A2AR). A1R are coupled to Gi/o and A2AR to Gs/olf protein subtypes, respectively, and their activation leads to inhibition and activation of neural excitability and to inhibition and facilitation of neurotransmitter release, respectively (Sebastiao and Ribeiro, 2009). A1R are widespread in the brain while A2AR are mostly concentrated in the striatum (Sebastiao and Ribeiro, 2009). In the striatum A2AR can be found both presynaptically and postsynaptically. Presynaptically they are localized in cortico-striatal glutamatergic terminals, where tey form heteromers with A1R, and postsynaptically they are localized in the dendritic spines of the striatopallidal neurons, where they form heteromers with D2R (Ciruela et al., 2006; Bonaventura et al., 2015; Ferré et al., 2016). We have previously shown that both brain iron deficiency in rodents and iron chelation in cells in culture produce a significant functional upregulation of A2AR (Gulyani et al., 2009; Quiroz et al., 2010). In rats with brain iron deficiency, striatal A2AR upregulation was observed both post and presynaptically, as demonstrated by an increased efficacy of an A2AR antagonist to induce locomotor activation and to block MAPK activation and motor output induced by cortical electrical stimulation, respectively (Quiroz et al., 2010). We therefore hypothesized that presynaptic striatal A2AR upregulation could be involved in the pathophysiology of the alterations of sensorimotor integration in RLS and represent a new therapeutic target for this disorder (Quiroz et al., 2010). But the possible A2AR upregulation in RLS still needs to be determined. Furthermore, to our knowledge, alterations in A1R density or function in RLS have not been addressed either in human patients or in the experimental animal. The present study was initially aimed at characterizing

A1R and A2AR density and function in a possibly improved rodent model of RLS, by analyzing the effects of more moderate but sustained ID diet in postweaning mice. Three different experimental preparations, long-term (22 weeks) severe or moderate ID diets (3 or 7 ppm iron) in mice and short-term (3 weeks) severe ID diet (3 ppm iron) in rats, demonstrated a significant downregulation of A1R, concomitant to D2R downregulation. On the other hand, A2AR upregulation was only observed with severe ID diets both in rats and mice. The data indicate A1R downregulation as a plausible key pathophysiological mechanism underlying PLMS and hyperarousal in RLS.

2. Materials and methods

2.1. Animals

All animal experiments were performed in accordance with the National Institutes of Health Animal care guidelines and the animal research was approved by the NIDA IRP Animal Care and Use Committee (protocol #: 12-BNRB-73). Thirty six male C57/BL6J mice (The Jackson Laboratory, Bar Harbor, ME) aged 3 weeks were grouped in three different diets. The control group was fed with a diet containing an essential amount of iron (48 ppm iron, TD.110846, Harlan-Teklad, Madison, WI) and the two other groups received ID diets, either a diet containing 7 ppm iron (TD.08655) or 3 ppm iron (TD.09127) for 22 weeks. The other contents of the diet were the same. Forty male Sprague-Dawley rats (Charles River

Laboratories, Wilmington, MA) aged 3 weeks were grouped in two different diets. The control group was fed with the diet containing an essential amount of iron (TD.110846) and the other group received the ID diet containing 3 ppm iron (TD.09127) for 3 weeks. All animals had continuous access to food and water and were maintained on a 12:12 h light/dark cycle (lights on at 06:00 h), with the room temperature maintained at 25±1 °C.

2.2. Blood serum iron analysis with long-term ID diets

In mice, the hematocrit was determined from blood samples drawn at weeks 4, 8, 12, 18 and 22 from the beginning of the diet. Blood was collected by ocular bleeding into EDTA tubes and kept on ice. Hematocrit values were determined using a Micro-Hematocrit Reader after centrifugation of blood samples in heparinized microcapillary tubes. The last blood samples were taken from the severed neck after euthanasia by isoflurane and decapitation, at the end of the 22-week diet period. Total serum iron and unsaturated iron binding capacity (UIBC) were measured using the Ferrozine based method (Equal Diagnostics; Exton, PA) and ferritin was measured using a latex-enhanced immunoturbidimetric assay (Kamiya Biomedical Company, Seattle, WA), on a Cobas Fara II robotic chemical analyzer (Roche; Basel, Switzerland). Total iron binding capacity (TIBC) was calculated by adding serum iron and UIBC values. Transferrin saturation was calculated as serum iron/TIBC x 100%.

2.3. Western blotting

In mice, after euthanasia by isoflurane and decapitation, brains were removed, frozen in dry ice-cold isopentane and stored at −80°C. Subsequently, 1 mm-thick coronal brains sections were cut at the level of 2 to 3 mm anterior to bregma, to include M1 and M2 frontal cortical regions and at the level of 0 to 1 mm anterior to bregma, to include the striatum. Bilateral punches from frontal cortical and striatal regions were taken from the corresponding tissue section with a 16-gauge sampling needle, kept frozen on dry ice, and stored at −30°C until processed. Tissue punches were sonicated for 10–15 s in 200 µl of 1% sodium dodecyl sulfate (SDS) dissolved in deionized ultrapure water. Protein concentration was determined by light absorbance using a bicinchoninic acid assay kit (Pierce, Rockford, IL) and samples were diluted with 1% SDS to equalize protein concentrations in each sample. Loading buffer (16% glycerol, 20% β-mercaptoethanol and 0.05% bromophenol blue) was added to each sample (sample to loading buffer ratio: 3:1) before heating at 95°C for 10 min. Proteins were separated by SDS-PAGE (10% polyacrylamide Tris-HCl resolving precast gel, Bio-Rad, Hercules, CA) for 3–4 h at 100 V. For each electrophoresis run, 10 µl of sample (containing 4–5 µg of total protein) were loaded in each well, and increasing amounts of protein pooled from the control samples were also loaded and electrophoresed to yield a five-point standard curve. Proteins were transferred electrophoretically during 7 min to polyvinylidene difluoride low fluorescence membranes (iBlot transfer kit, Invitrogen, Carlsbad, CA). Membranes were then

dried overnight, rewetted and washed four times for 15 min in blocking buffer (2% bovine albumin in phosphate-buffered saline plus 0.05% Tween 20; pH 7.4) and incubated overnight at 4°C with primary antibody diluted in blocking buffer plus 0.01% sodium azide. Primary antibodies were: mouse monoclonal anti-TFR (1:1000 dilution; Invitrogen), mouse monoclonal anti-A2AR (1:1000 dilution; Upstate, Lake Placid, NY), rabbit polyclonal anti-D2R (1:1000 dilution, EMD Millipore, Darmstadt, Germany) and rabbit polyclonal anti-A1R (1:1000 dilution; Thermo Scientific; Wilmington, Laurel, DE). After four washes for 15 min in blocking buffer, blots were incubated for 2 h at room temperature with goat anti-mouse IgG Cy5 or goat anti-rabbit IgG Cy5 (1:5000 dilution; GE Healthcare). Finally, blots were washed six times for 10 min in PBS, allowed to dry and scanned in a Typhoon laser scanner (GE Healthcare). The resulting blot images were analyzed and quantified with ImageQuant software (GE Healthcare). The amount protein of interest in each sample was interpolated from the band intensities of the standard curves. Quantified band intensities were always within the range of the standard curve. In each Western blot, all values were normalized (as percentage of control) with respect to the standard curve.

2.4. Radioligand saturation experiments

In rats, after euthanasia by overdose of Equitesin (10 ml/kg; 4.44 g chloral hydrate, 0.972 g Na pentobarbital, 2.124 g MgSO4 44.4 ml propylene glycol, 12 ml ethanol, and distilled H_2O up to 100 ml of final solution; NIDA Pharmacy) and

decapitation, the brains were quickly removed on ice and the frontal cortex and the striatum dissected out and homogenized in 50 mM Tris-HCl (pH 7.4) containing a protease inhibitor cocktail (1:1000; Sigma-Aldrich Co, Saint Louis, MO). Membranes were obtained by centrifugation at 48,000 g (50 min, 4°C) and washed 2 times in the same conditions. Protein was quantified by the bicinchoninic acid method (Pierce). Pellets were resuspended and membrane suspensions (0.1 mg of protein/ml) were incubated in 50 mM Tris-HCl (pH 7.4) containing 2 I.U./ml of adenosine deaminase (ADA, E.C.3.5.4.4, Roche), 10 mM $MgCl_2$ and increasing concentrations of the A1R antagonist [^{3}H]DPCPX (120 Ci/mmol, ARC, Saint Louis, MO) or the A2AR antagonist [^{3}H]ZM241385 (50 Ci/mmol, ARC) during 2 h at RT. Non-specific binding was determined in the presence of 10 mM of non-labeled A1R antagonist 8-cyclopentyl-1,3-dimethyl-xanthine (CPT; Sigma-Aldrich) or non-labeled ZM241385 (Sigma-Aldrich) for A1R or A2AR, respectively. For D2R binding, membrane suspensions (0.1 mg of protein/ml) were incubated in 50 mM Tris-HCl (pH 7.4) 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$ and increasing concentrations of the D2R-like antagonist [^{3}H]raclopride (78.1 Ci/mmol, PerkinElmer, Boston, MA). Non-specific binding was determined in the presence of 10 mM of non-labeled raclopride (Tocris, Bristol, UK). In all cases, free and membrane-bound ligand were separated by rapid filtration on 500-µl aliquots in a 96-well plate harvester (Brandel, Gaithersburg, MD) and washed with 2 ml of ice-cold Tris-HCl. Microscint-20 scintillation liquid (65 µl/well, PerkinElmer) was added to the filter plates, plates incubated overnight at RT and radioactivity counts were determined in a MicroBeta2 plate counter (PerkinElmer) with an efficiency of 41%. Saturation curves were fitted to one-site binding

hyperbolic curves using Prism 4 (GraphPad Software, La Jolla, CA).

2.5. Locomotor activity

In mice, by the end of the 22-week diet period, and in rats, at the end of the 3-week diet period, spontaneous exploratory and drug-induced locomotion were recorded using an open field arena (43 cm width, 43 cm length and 27 cm height; Med Associates, Georgia, VT) inside a sound proof box. The chamber received constant illumination (110 v, 5 w). Horizontal movements were monitored by two sets of pulsed-modulated infrared photobeams, which allows automatic measurement of total distance traveled. Experiments were performed between 10:00 am and 3:00 pm and locomotion was recorded during the first 90 min of exploratory activity when the animal was introduced in the chamber, followed by another 60 min after the systemic (i.p.) administration of the corresponding adenosine receptor antagonist. The drug was administered at the end of the first 90 min and the animal was placed back to the arena for continuous recording without interruption. The A2AR antagonist (E)-1, 3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (istradefylline or KW-6002; Sigma, St Louis, MO, USA), was suspended in a solution of 8% TWEEN80 and 92% ddH_2O. The A1R antagonist CPT and the non-selective adenosine receptor antagonist caffeine were dissolved in sterile sodium chloride solution, 0.9%. All mice received the three different drug treatments during the last two weeks of the 22-weeks period of ID or control diet following a counterbalanced schedule with 72 h of washout between

each treatment. Another 72-h washout period was allowed before euthanasia and subsequent Western blot experiments. Rats were only used once, for one of the treatments.

2.6. Statistical analysis

In mice, the comparison of the effect of the different diets (control, 3-ppm and 7-ppm) on hematocrit on different time points during the 22 weeks after the beginning of the diet was analyzed by a two-way ANOVA followed by Newman-Keuls posthoc test. Also in mice, one-way ANOVA followed by Newman-Keuls posthoc test was used for comparisons of the effects of the different diets on serum iron values and ferritin, on brain densities for Western blot experiments and on adenosine antagonist-induced locomotor activity. In rats, non-paired *t* test was used to compare the number of radioligand binding sites (B_{max}) and affinities (K_D) and on adenosine antagonists-induced locomotor activation between animals subjected to the ID diet and controls. GraphPAD Prism Software, version 5 (San Diego, CA) was used for the statistical analysis.

3. Results

3.1. Hematocrit, serum iron and ferritin and brain TFR density in mice with long-term ID diets

Hematocrit was significantly lower in the 3-ppm and 7-ppm groups compared to the control group 4 weeks after the beginning of the ID diet. However, longitudinal measurements showed hematocrit normalization in the 7-ppm group at 8 weeks up to the last measurement, at 22 weeks (Fig. 1A). On the other hand, hematocrit in the 3-ppm group remained significantly lower compared to the control group up to 22 weeks (Fig. 1A). Serum iron, ferritin, transferrin saturation, TIBC and UIBC average values from the last blood sample in animals from the control, 3-ppm and 7-ppm groups are shown in Fig. 1B-F. Serum iron values were significantly lower in both 3-ppm and 7-ppm groups compared to control, without significant differences between the values of the 3-ppm and 7-ppm groups; serum ferritin and transferrin saturation values were significantly lower in the 3-ppm and 7-ppm compared to control and the values of the 3-ppm group were significantly lower compared to the 7-ppm group; TIBC values were significantly higher in the 3-ppm group but not in the 7-ppm group as compared to the control group; finally, UIBC values were significantly higher in both the 3-ppm and the 7-ppm groups compared to the control group and in the 3-ppm as compared to the 7-ppm group. TFR density in brain tissue was used as a measure of severe brain iron deficiency (Lok and Loh, 1988; Han et al., 2003). Both in striatum and cortex, TFR density was found significantly increased in the 3-ppm, but not in the 7-ppm, as compared to the control group (Fig. 2). In summary, both diets were efficient at reducing serum iron and ferritin levels, but only the more severe diet (3-ppm ID diet) was associated with a significantly low hematocrit and severe brain iron deficiency.

3.2. Density of adenosine and dopamine receptors in mice with long-term ID diets

Western blot experiments showed that both the 3-ppm and 7-ppm groups were associated with a significant decrease, of about 20% compared to the control group, in striatal D2R density, while only the 3-ppm group was associated with a significant increase, of about 30% compared to the control group, in striatal A2AR density (Fig. 3A,B). In the striatum and cortex, A1R density was also significantly decrease, by about 20% compared to the control group, in both the 3-ppm and 7-ppm groups (Fig. 3C,D). In summary, both ID diets were associated with downregulation of D2R and A1R, while only the more severe diet (3-ppm ID diet) was associated with striatal A2AR upregulation.

3.3. Dopamine and adenosine antagonist binding sites in the rats with short-term ID diet

Saturation experiments with the A1R antagonist [^{3}H]DPCPX, the A2AR antagonist [^{3}H]ZM241385 and the D2R antagonist [^{3}H]raclopride were performed in our previously characterized brain iron deficiency rat model with a short-term (three weeks) of severe ID diet (3-ppm). The results showed a significant decrease in the number of A1R antagonist binding sites (B_{max} values) in the cortex and striatum (27% and 29%, respectively), a significant increase in striatal A2AR antagonist binding sites (25%) and a significant decrease in striatal D2R antagonist binding sites (35%) as compared with rats that received the control diet (Fig. 4).

The affinity of the three ligands was not significantly modified: K_D values of [^{3}H]DPCPX in the cortex for brain iron deficient rats and controls were (in means ± S.E.M) 0.45 ± 0.04 nM and 0.45 ± 0.05 nM, respectively; K_D values of [^{3}H]DPCPX in the striatum for brain iron deficient rats and controls were 0.58 ± 0.06 nM and 0.39 ± 0.05 nM, respectively; K_D values of [^{3}H]ZM241385 for brain iron deficient rats and controls were 0.63 ± 0.05 nM and 0.71 ± 0.09 nM, respectively; and K_D values of [^{3}H]raclopride for brain iron deficient rats and controls were 3.6 ± 0.9 nM and 2.8 ± 0.7 nM, respectively.

3.4. Adenosine receptor antagonist-induced locomotor activity in mice with long-term ID diets and rats with short-term ID diet

In our previous study in rats after a short-term severe ID diet (with the same protocol used in the present study), an increase in the locomotor activity in response to and A2AR antagonist paralleled the upregulation of striatal (postsynaptic) A2AR (Quiroz et al., 2010). The same correlation could be observed in mice with long-term ID diets. Thus, the A2AR antagonist KW-6002 (1 mg/kg, i.p.) only produced a significantly higher locomotor activation with the most severe ID diet (3 ppm), associated with the increased striatal density of A2AR (Fig. 5A). On the other hand, the A1R antagonist CPT (4.8 mg/kg, i.p.) and the non-selective A1R-A2AR antagonist caffeine (10 mg/kg, i.p.) produced locomotor activation in the three experimental groups, but without significant differences (Fig. 5A). Since the lack of correlation between A1R density and locomotor activation by CPT could be

related to the effects of sequential administration of adenosine antagonists (see Discussion), additional independent experiments with CPT and caffeine were performed in rats with short-term severe ID diet. Rats received only one drug treatment, CPT 4.8 mg/kg or caffeine 10 mg/kg. These were previously shown as the minimal doses with maximal effect in the same animal strain (Karcz-Kubicha et al., 2003). Under these conditions CPT produced a significant decrease in its ability to produce locomotor activity in the rat with severe ID diet as compared to control, while caffeine did not show significant differences (Fig. 5C).

4. Discussion

Induction of brain iron deficiency in rodents during the postweaning period is a widely used model that reproduces some of the key dopaminergic neurochemical abnormalities observed in RLS patients. At the behavioral level it does not show motor disturbances that would mimic the PLMS of RLS, although it demonstrates a circadian sleep architecture that mimics the hyperarousal in RLS (Dean et al., 2006; Ferri et al., 2014). This model usually involves a relatively short period of ID diet, from 3 to 8 weeks depending on the study, and, under these conditions, a significant downregulation of striatal D2R and upregulation of A2AR have been described (Erikson et al., 2001; Unger et al., 2008; Connor et al., 2009; Gulyani et al., 2009; Quiroz et al., 2010). Aiming initially at evaluating the role of a less severe but long-term ID diet (7 ppm for 22 weeks) in postweaning mice, and comparing with a long-term and severe ID diet (3 ppm for 22 weeks), a dissociation

of the effects of iron deficiency on the density of striatal D2R and A2AR was found with Western blot experiments. Striatal D2R, but not A2AR, was significantly downregulated after both moderate and severe long-term diets, while the more severe ID diet was needed to produced A2AR upregulation. Significantly, A1R showed the same alterations that D2R, with a decrease in striatal and also cortical density upon both long-term ID diets, indicating a higher sensitivity of A1R *versus* A2AR to brain iron deficiency.

Brain iron deficiency-induced downregulation of A1R was confirmed with radioligand-binding experiments in the rat with short-term (3 weeks) and severe ID diet. Saturation experiments with selective radiolabeled antagonists provided clear demonstration of a significant reduction in the number of A1R in the two analyzed brain areas, striatum and cortex, without changes in affinity. The same results, a decrease in the number of receptors without a change in affinity, were observed in striatal tissue with D2R, as previously reported (Erikson et al., 2001). To our knowledge, this was also the first time that saturation experiments were used to determine differences in A2AR ligand binding. In agreement with the present and previous studies using Western blotting, striatal A2AR number was significantly increased and, additionally, no changes in affinity were found.

Adenosine antagonist-induced locomotor activation was used as a functional correlate of the biochemical changes of striatal postsynaptic A1R and A2AR (Ferré et al., 1994; Popoli et al., 1996, Quiroz et al., 2010; Orru et al., 2011). In agreement with our previous studies in rats (Quiroz et al., 2010), locomotor activity induced by an A2AR antagonist was only significantly increased in mice with the long-term ID

diet inducing A2AR upregulation. On the other hand, locomotor activating doses of the A1R antagonist CPT or caffeine did not show a differential effect among controls and the two long-term ID diets in mice. Nevertheless, CPT produced a significantly lower locomotor activation in rats with severe short-term ID diet, while caffeine did not show significant differences. Since the locomotor-activating effects secondary to A1R receptor blockade but not A2AR blockade develop strong tolerance (Karcz-Kubicha et al., 2003), cross-tolerance between CPT and caffeine due to the sequential counterbalanced adenosine receptor antagonist administration could have masked a differential effect due to A1R downregulation in mice. The lack of differences with the locomotor activation induced by caffeine in rats could be attributed to the opposite influence of A1R downregulation and A2AR upregulation, in view of the reported dependence on A1R- and A2AR-mediated blocking effects upon caffeine acute administration (Karcz-Kubicha et al., 2003; Antoniou et al., 2005).

It is important to emphasize that locomotor activation induced by adenosine receptor antagonists are mediated by postsynaptic striatal A1R and A2AR (Ferré et al., 1994; Popoli et al., 1996, Quiroz et al., 2010; Orru et al., 2011). In a previous study we showed that brain iron deficiency in rats induces upregulation of both postsynaptic and presynaptic A2AR localized in corticostriatal glutamatergic terminals (Quiroz et al., 2010). We then proposed that presynaptic A2AR upregulation could constitute a pathophysiological mechanism of PLMS in RLS (Quiroz et al., 2010). Presynaptic A2AR upregulation could increase the sensitivity of corticostriatal terminals to release glutamate, decreasing the filtering of multiple

cortical signals that converge in the striatum and therefore impairing striatal sensory-motor integration. Presynaptic A2AR antagonists (Orru et al., 2011) could then constitute a new therapeutic approach for the PLMS component of RLS. In fact, in a recent study, an A2AR antagonist was found significantly effective in a non-human primate model of neuroleptic-induced akathisia (Bleikardt et al., 2014). On the other hand, downregulation of both postsynaptic and presynaptic striatal A1R could be involved in the pathogenesis of PLMS in RLS. Postsynaptic A1R form heteromers with D1R and their activation or blockade leads to a respective selective decrease or increase in D1R-mediated activation of D1R-expressing striato-nigral neurons (Ferré et al., 1997; Ferré, 2008, 2010). Presynaptic A1R form heteromers with A2AR in the striatal glutamatergic terminals and their activation or blockade leads to a respective decrease or increase in glutamate release (Quarta et al., 2004; Ciruela et al., 2006; Ferré, 2008, 2010). Downregulation of both pre and postsynaptic A1R should lead to a significant decrease in the brake imposed by adenosine on the activity of the striato-nigral neurons, which could promote PLMS.

In addition, downregulation of A1R in the cortex, and perhaps in other unexplored brain areas, is a plausible mechanism of hyperarousal in RLS. Adenosine is a main mediator of sleepiness following prolonged wakefulness, when it accumulates in the extracellular space of basal forebrain, cortex and hypothalamus (McCarley, 2007; Ferré, 2010). This accumulation leads to: A1R-mediated inhibition of the cells of origin of the corticopetal basal forebrain system (McCarley, 2007; Ferré, 2010); inhibition of corticofugal neurons from the prefrontal cortex that target the origin of pontine ascending arousal systems (Van Dort et al., 2009), also

mediated by A1R; and inhibition of hypothalamic histaminergic and orexinergic ascending arousal systems, with both A1R and A2AR being involved (McCarley, 2007; Ferré, 2010). A brain iron deficiency-mediated A1R downregulation, particularly in the basal forebrain, cortex and hypothalamus, can then constitute a major pathophysiological mechanism involved in the hyperarousal and sleep disturbances of RLS. In fact, several studies indicate that A1R is a marker of the homeostatic sleep responses, the need for recovery of lost sleep, which involves the rebound sleepiness (operatively measured as propensity to fall asleep) upon acute sleep deprivation and the cumulative sleepiness upon chronic sleep deprivation (Bjorness et al., 2009, 2016; Kim et al., 2012). In fact, both acute and chronic sleep deprivation lead to an increase of A1R density in the brain, including cortex and striatum (Elmenhorst et al., 2007, 2009; Kim et al., 2012, 2015).

The differential results on A2AR density observed between the two long-term ID diets in mice suggest that further work will be required to establish the most appropriate animal model for RLS. PET experiments in RLS patients should now determine the existence of downregulation of A1R, upregulation of A2AR or both and, therefore, adenosine receptors could constitute new targets for this very prevalent neurological disorder.

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Figure Legends

Fig. 1. A. Hematocrit values during the 22 weeks of severe (3-ppm) or mild (7-ppm) ID-diet or control diet in mice. Results are means ± S.E.M. of 12 animals/group. ***: $p<0.001$ compared to the control (same time-period); two-way ANOVA followed by Newman-Keul's posthoc test. B-F. Serum iron (B), serum ferritin (C), transferrin saturation (D), Total iron binding capacity (TIBC) (E) and unsaturated iron binding capacity (UIBC) (F) values after 22 weeks of severe (3-ppm) or mild (7-ppm) ID-diet or control diet. Results are means ± S.E.M. of 12 animals/group. ** and ***: $p<0.01$ and $p<0.001$ compared to the control group, respectively; # and ###: $p<0.05$ and $p<0.001$ compared to the 3-ppm group, respectively; one-way ANOVA followed by Newman-Keul's posthoc test.

Fig. 2. TFR density in cortical (A) or striatal (B) tissue after 22 weeks of severe (3-ppm) or mild (7-ppm) ID-diet or control diet in mice. Results are means ± S.E.M. of 12 animals/group. * and ***: $p<0.05$ and $p<0.001$ compared to the control group, respectively; ###: $p<0.001$ compared to the 3-ppm group; one-way ANOVA followed by Newman-Keul's posthoc test.Bottom images show representative Western blots.

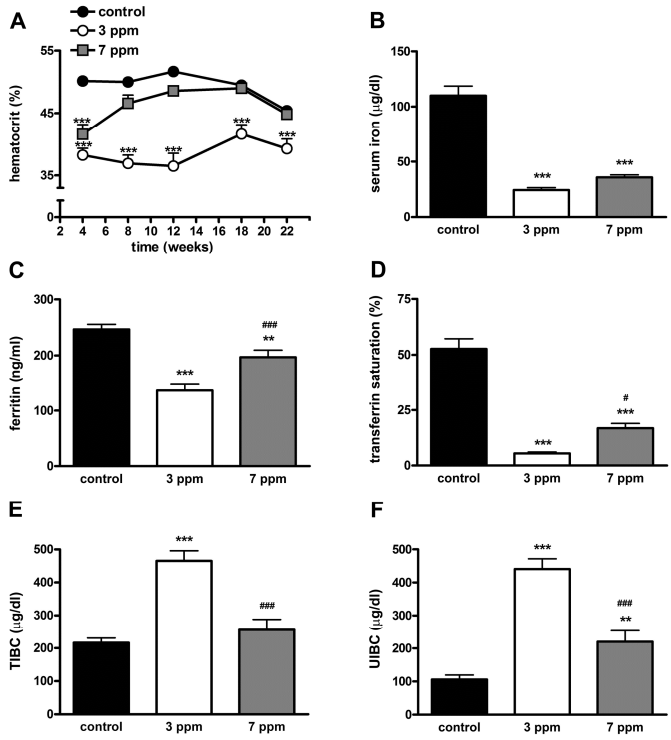
Fig. 3. A-D. A2AR (A), D2R (B) and A1R (D) density in striatal tissue and A1R density in cortical tissue (C) in mice after 22 weeks of severe (3-ppm) or mild (7-ppm) ID-diet or control diet. Results are means ± S.E.M. of 6-12 animals/group. *, **: $p<0.05$

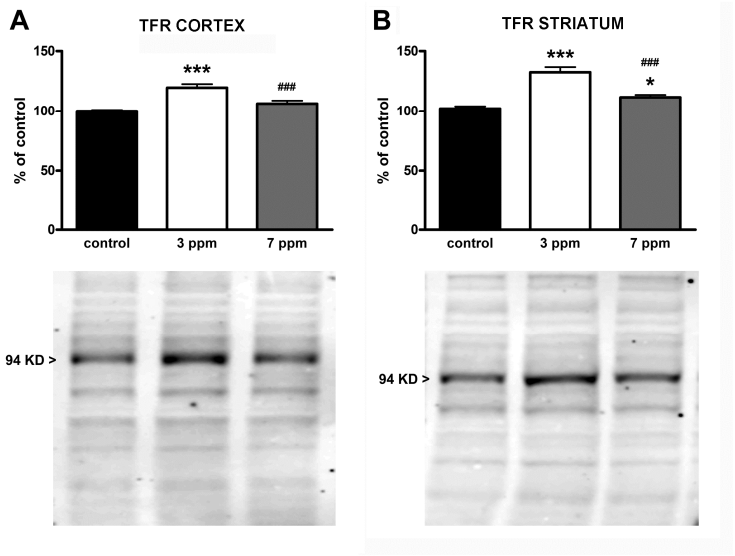
and p<0.01 compared to the control group, respectively; one-way ANOVA followed by Newman-Keul's posthoc test. Bottom images show representative Western blots.

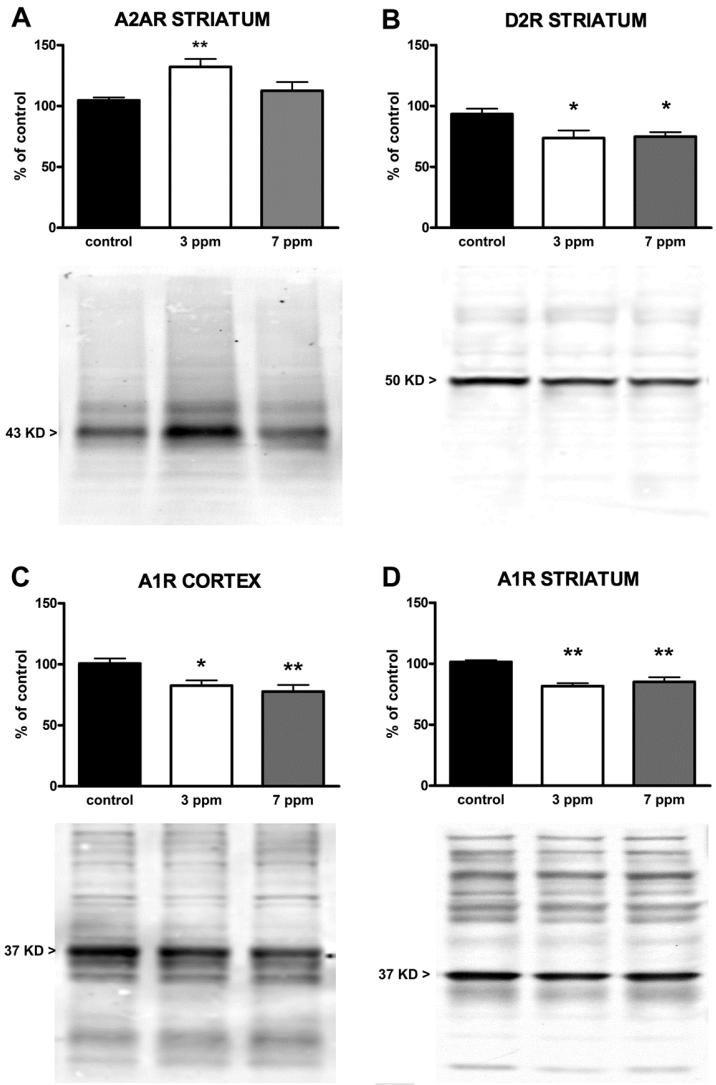
Fig. 4. A-D. Representative saturation experiments with the A1R antagonist [^{3}H]DPCPX in cortical (A) and striatal (B) rat tissue and with the A2AR antagonist [^{3}H]ZM241385 (C) and the D2R antagonist [^{3}H]raclopride (D) in rat striatum after 3 weeks of a severe ID diet (3 ppm) and controls. E. B_{max} values from all saturation experiments; results are means ± S.E.M. of 3-5 animals/group. *, **: p<0.05 and p<0.01 compared to the control group, respectively; non-paired *t* test.

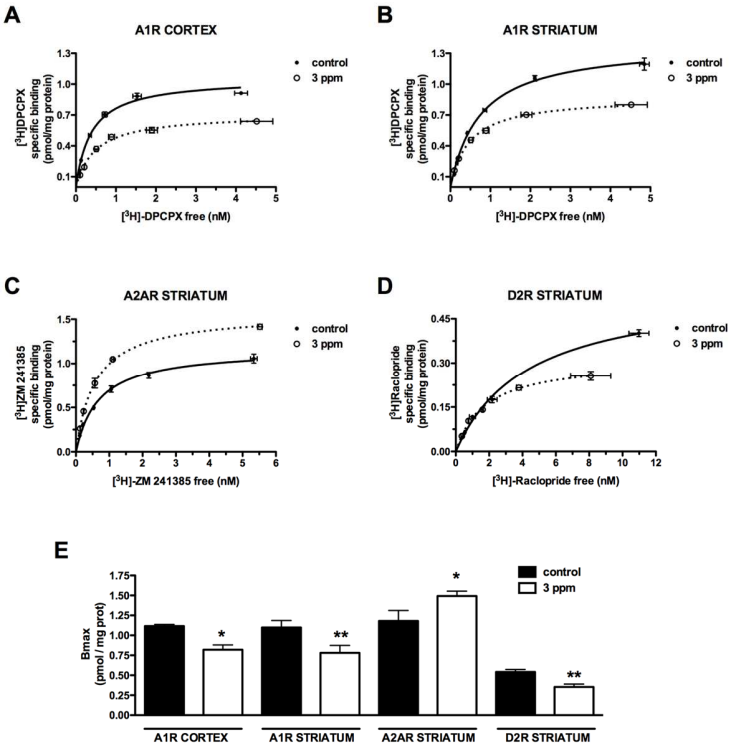
Fig. 5. A. Average locomotor activity in mice during 60 min after systemic (i.p.) administration of the A2AR antagonist KW-6002 (1 mg/kg), the A1R antagonist CPT (4.8 mg/kg) or caffeine (10 mg/kg). Broken line indicates the average level of locomotor activity at the end of the habituation period, which did not differ among the three groups. Results represent accumulative ambulatory counts and are expressed as means ± S.E.M. (12 animals per group). All mice were tested with the three drugs (with a counterbalanced schedule with 72 h of washout between each treatment) by the end of a 22-weeks period of severe (3-ppm) or mild (7-ppm) ID-diet or control diet. **: p<0.01 compared to the control group; one-way ANOVA followed by Newman-Keul's posthoc test. B. Average locomotor activity in rats during 60 min after systemic (i.p.) administration of the CPT (4.8 mg/kg) or caffeine (10 mg/kg). Broken line indicates the average level of locomotor activity at the end of the habituation period, which did not differ among the three groups. Results

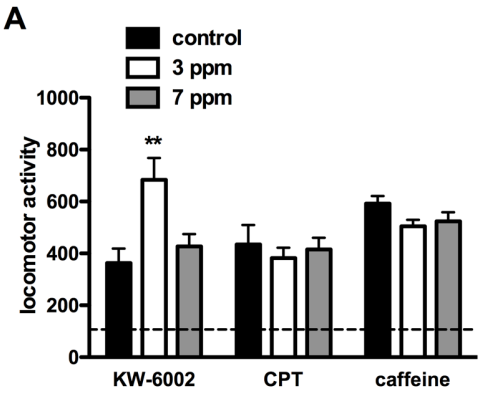
represent accumulative ambulatory counts and are expressed are means ± S.E.M. (6 animals per group). Rats were only used once, for one of the treatments.

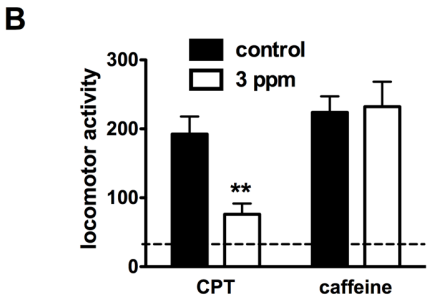












Highlights

- Brain iron deficiency (BID) in rodents is a valid pathophysiological model of RLS
- BID was previously shown to cause upregulation of adenosine A_{2A} receptors (A2AR)
- We demonstrate that BID also causes downregulation of adenosine A_1 receptors (A1R)
- A1R downregulation can constitute a key pathophysiological factor in RLS.