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Diminished iron concentrations increase adenosine A_{2A} receptor levels in mouse striatum and cultured human neuroblastoma cells

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Abstract

Brain iron insufficiency has been implicated in several neurological disorders. The dopamine system is consistently altered in studies of iron deficiency in rodent models. Changes in striatal dopamine D₂ receptors are directly proportional to the degree of iron deficiency. In light of the unknown mechanism for the iron deficiency-dopamine connection and because of the known interplay between adenosinergic and dopaminergic systems in the striatum we examined the effects of iron deficiency on the adenosine system. We first attempted to assess whether there is a functional change in the levels of adenosine receptors in response to this low iron. Mice made iron-deficient by diet had an increase in the density of striatal adenosine A_{2A} (A_{2A}R) but not A₁ receptor (A₁R) compared to mice on a normal diet. Between two inbred murine strains, which had 2-fold differences in their striatal iron concentrations under normal dietary conditions, the strain with the lower striatal iron had the highest striatal A_{2A}R density. Treatment of SH-SY5Y (human neuroblastoma) cells with an iron chelator resulted in increased density of A_{2A}R. In these cells, A_{2A}R agonist-induced cyclic AMP production was enhanced in response to iron chelation, also demonstrating a functional upregulation of A_{2A}R. A significant correlation ($r^2=0.79$) was found between a primary marker of cellular iron status (transferrin receptor (TfR)) and A_{2A}R protein density. In conclusion, the A_{2A}R is increased across different iron-insufficient conditions. The relation between A_{2A}R and cellular iron status may be an important pathway by which adenosine may alter the function of the dopaminergic system.

Keywords

CGS-21680; Desferrioxamine; Dietary iron deficiency; Adenosine receptors; Restless legs syndrome; SH-SY5Y

Introduction

In recent years, accumulating evidence has demonstrated that the adenosinergic receptor system may play a vital role in the etiology and pathology of several neurological disorders.

There are four subtypes of adenosine receptors found in most mammals, A₁, A_{2A}, A_{2B} and A₃ (Olah et al., 1995). These receptors are classified as rhodopsin-like G protein-coupled receptors (GPCRs). With respect to central nervous system disorders, the A_{2A} receptor (A_{2A}R) has been shown to be physiologically relevant in cerebral ischemia (Ongini et al., 1997), neurological disorders (Kafka and Corbett, 1996; and Popoli et al., 1998) and neurodegenerative processes, such as Parkinson's (Mandhane et al., 1999), Huntington's (Blum et al., 2003) and Alzheimer's disease (Dall'Igna et al., 2007).

Notably A_{2A}R is abundant throughout the central nervous system and shows a significant concentration in the striatum (Ongini et al., 1997). As the striatum is a brain region vital for fine motor control skills, the potential involvement of the adenosinergic system in movement disorders is likely. Hence, an A_{2A}R antagonist has been shown to be beneficial in association with L-DOPA in patients with Parkinson's disease (Jenner, 2005). Parkinsonian conditions are typically mediated by a significant disruption of dopaminergic receptor signaling; however, relief can be induced with the implementation of agents targeted to adenosine receptors. This functional interaction between adenosinergic and dopaminergic receptor signaling has been well studied (Ferre et al., 1997; Ferre et al., 2007; and Schiffmann et al., 2007). There are other central neurological disorders that also share a similar strong dopaminergic component in which this functional receptor synergy may also be relevant, including for example, restless legs syndrome (RLS).

RLS is a common sensory-motor disorder that affects between 2-3% of the population by severely disrupting both the quantity and quality of sleep (Allen et al., 2005). Insufficiency of brain iron has been identified as a primary neuropathological factor in several neurological disorders, including RLS (Earley et al., 2000). CSF analysis (Earley et al., 2000b; and Mizuno et al., 2005), MRI iron quantification (Allen et al., 2001; and Earley et al., 2006a), ultrasound imaging (Schmidauer et al., 2005), and autopsy tissue (Connor et al., 2003; and Connor et al., 2004) have all shown that CNS iron is diminished in RLS patients, particularly in the substantia nigra and nucleus putamen. Further support for the primary role of low brain iron in RLS is the improvement or resolution of symptoms with the use of oral or intravenous iron therapy (Nordlander, 1953; and O'Keeffe et al., 1994). Despite the primacy of brain iron insufficiency in central neuropathologies, the mechanisms by which this low iron state leads to the symptomatic clinical condition are not known.

The almost universal therapeutic benefits of levodopa in treating RLS (Hening et al., 1999) have implicated a role for the dopaminergic system, while therapeutic benefits of selective dopamine D₂₋₃ receptor (D₂₋₃R) agonists in RLS suggest the possibility of involvement of differential receptor subtypes in RLS (Paulus and Trenkwalder, 2006). At the present time, low brain iron in RLS has been posited as directly or indirectly leading to a change in the dopaminergic system that then leads to clinical symptoms (Allen, 2004). Indeed several results indicate that iron insufficiency modifies dopamine release, and expression of dopamine receptors, and the dopamine transporter (Nelson et al., 1997; Erikson et al., 2000; Erikson et al., 2001; and Wiesinger et al., 2007).

As previously stated, A_{2A}Rs are particularly concentrated in the striatum, where they can physically and functionally interact with D₂Rs (Ferre et al., 1997; Ferre et al., 2007; and Schiffmann et al., 2007). Given this strong association, it seems likely that the well-established iron insufficiency effects on D₂R would be accompanied by effects on A_{2A}R. Alternatively, A_{2A}R-D₂R heteromerization establishes a potential mechanism by which iron could influence one aspect of the dopaminergic system, by directly modifying the density or function of A_{2A}R. If either case were true, the results would provide new insights into the neurobiological effects of brain iron insufficiency and thus would further enhance our understanding of the biology underlying RLS. In this paper we evaluate our primary hypothesis that decreased

cellular iron concentrations would result in modulation of A_{2A}R functionality, which may provide a link of brain iron to neurological disorders such as RLS. As a first step, we examined the effects of iron insufficiency on A_{2A}R in the striatum, a region where current literature implicates a change in the D₂R in RLS, and in iron insufficiency rodent models. The positive results in animals, which are reported in this paper, led us to a more detailed examination of the effects of iron insufficiency on A_{2A}R in a human neural cell line.

Materials and methods

Iron-deficient (ID) rodent model

Three-week-old male C57/BL6J mice were purchased from Jackson Laboratories and divided into two groups. The control group was fed a diet containing an essential amount of iron (48 ppm iron, TD.80394, Harlan-Teklad, Madison, WI) and the iron deficient (ID) group was fed a diet containing a low iron concentration (4 ppm iron, TD.80396) for 8 weeks. The contents of the diet were otherwise exactly the same. All mice had continuous access to food and water and were maintained on a 12:12 h light/dark cycle (lights on at 0600 h), with the room temperature maintained at 25±1 °C. The iron status of the mice was monitored by measuring hemoglobin (Hb). Blood samples were collected from retro-orbital sinus and placed in EDTA-heparinized centrifuge tubes to prevent clotting. Hemoglobin was measured calorimetrically by the cyanomethemoglobin method. At the end of 8 weeks mice were euthanized by using isoflurane anesthesia followed by decapitation. The striatum and cortex were carefully dissected out and snap frozen on dry ice. The brain tissues were stored at -80 °C until analyses were performed.

Recombinant inbred mice

We used female mice from two different strains (strain 40 and 1) of the BXD/Ty recombinant inbred strain panel generated from C57BL/6 and DBA/2 parental strains (Morse et al., 1979). These strains were chosen as they were at extremes in their striatal iron concentrations. Strain 40 mice had a striatal iron concentration of 12±4.4 µg/g tissue while strain 1 had an iron concentration of 22±8.6 µg/g as measured by atomic absorption spectroscopy (Nelson et al., 1997). We had selected female mice over males because females showed the greatest differences in striatal iron while maintaining similar peripheral iron measures. All mice were obtained from our BXD/Ty breeding colony at the Pennsylvania State University. At 21 days after birth, mice were separated by sex and were housed 2-4/cage. Breeders and offspring had *ad libitum* access to water and food (Purina Mills Lab diet #5001; St. Louis, MO). Mice were maintained on a 12:12 h light/dark cycle (lights on at 0600 h). The room temperature was maintained at 25±1 °C and mice were euthanized at 120±3 days of age. The striatum was dissected out and snap frozen on dry ice. The brain tissues were stored at -80 °C until analyses were performed. All experimental protocols were conducted in accordance with the National Institutes of Health Animal Care guidelines and were approved by the Penn State Institutional Animal Care and Use Committee.

Cell culture

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium containing 10% horse serum (Invitrogen, Carlsbad, CA) and 5% fetal clone II (Hyclone, Logan, UT). Cells were maintained in humidified 37 °C chambers with 5% CO₂. Cells were plated at a density of about 10⁵ cells/100 mm dish and were treated with experimental agents about 48 h later. Cells were treated with various concentrations of Desferrioxamine (DFO) (Sigma, St. Louis, MO) for the dose-response analysis, and for different time periods for time course analysis.

Immunoblot analysis

For immunoblot analysis, protein extracts from cultured cells and mouse striatum were prepared by solubilizing the cells and brain tissue in SDS-PAGE sample buffer. Protein concentrations were determined using a BCA assay kit from PIERCE (Woburn, MA) with a BSA standard. Samples were prepared with protein loading buffer and heated to 70 °C for 10 min instead of boiling for immunoblot analysis. All protein samples were resolved (50-100 µg per lane) in a 10% SDS-polyacrylamide gel (NuPage gels/Invitrogen) at 100 V. Proteins were transferred to 0.2 µm PVDF membrane, and membranes were blocked with 4% nonfat milk in Tris-buffered saline-Tween 20 for 2 h. Membranes were incubated overnight in primary antibody in 4% nonfat milk. Membranes were washed and then incubated for 1 h in the presence of a 1:5000 dilution of secondary antibody (anti-mouse or anti-rabbit IgG) conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). Reaction products were visualized by using an ECL Western Blot Detection kit (GE Healthcare, Piscataway, NJ). The band densities were quantified using Image J software and final mean and S.D. were calculated after band densities were normalized to actin bands. The following antibodies were used: anti-A_{2A}R (1:1000, Upstate/Millipore, Billerica, MA), anti A1R (1:1000, Affinity Bioreagents, Golden, Co.), anti-HSP-70 (1:1000, Stressgen, Ann Arbor, MI), anti-phospho-SAPK/JNK (1:1000, Cell signaling, Danvers, MA) anti-actin (1:5000; Sigma), and anti-TfR (1:1000, Zymed/Invitrogen).

Immunocytochemistry

SH-SY5Y cells were plated onto coverslips in 24-well plates 2 days prior to treatment. After 24 h treatment with DFO, cells were fixed with 4% paraformaldehyde for 20 min, blocked with 5% BSA for 1 h, followed by overnight incubation with rabbit anti-A_{2A}R antibody (1:50, VC-21) (Canals et al., 2005). The cells were washed in PBS and incubated for 1 h in FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted in PBS (1:500). They were then washed with PBS and mounted on microscope slides in PermaFluor mounting medium (Immunon, Pittsburgh, PA). No detergent was added in the wash buffer so that cells remained impermeable to antibody. Fluorescence images were acquired by confocal microscopy (LSM-510; Zeiss, Oberkochen, Germany) by using a 40× objective in a scanning mode for detection of fluorescein (488 nm excitation and 510 nm emission). The average pixel intensity for each cell was quantified. Forty-four untreated and 40 iron-chelated cells were analyzed.

RNA extraction and real-time PCR

RNA was isolated using Trizol (Invitrogen) and purified with an RNA Micro Kit (Qiagen, Valencia, CA). Following treatment with DNase I, RNA was quantified and equal amounts were retrotranscribed using random hexamers and the SuperScript First Strand Synthesis System (Invitrogen Life Technologies). Real-time PCR analysis was performed with an ABI Prism 7000 sequence detection system and Sybr® Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Each reaction included 3 µl of diluted (1:3) cDNA and was performed in duplicate. PCR was performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers used in this study were as follows: murine adenosine A_{2A}R (NM_009630), 5'-TTG CCA TCG ACA GAT ACA TCG CCA-3' and 5'-AGC AAA TCG CAA TGA TGC CCT TCG-3'; murine GAPDH, 5'-TGC ACC ACC AAC TGC TTA-3' and 5'-GAT GCA GGG ATG ATG TTC-3'.

cAMP measurements

Accumulation of cAMP was measured with the Cyclic AMP Enzyme immunoassay System (BIOMOL, Plymouth, PA) as described in the manual from the manufacturer. SH-SY5Y cells (80% confluent) were serum-depleted for 12-16 h supplemented with 2 IU/ml of adenosine

deaminase (ADA; Roche, Basel, Switzerland). After 16 h, the medium was replaced with fresh medium and cells were incubated with 50 μ M Zardaverine as phosphodiesterase inhibitor for 15 min at 37 °C. The A_{2A}R agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS 21680, 0-100 nM; Sigma) (Karcz-Kubicha et al., 2003) was added and incubated for 30 min at 37 °C. Zardaverine was purchased from Tocris (Bristol, UK). CGS 21680 and ZM241385 were initially dissolved in DMSO (concentration of DMSO in the final dilution was <0.01%). cAMP levels were measured in cell culture samples according to the manufacturer's instructions. The absorbance was measured using a Thermo Multiskan Ascent spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The individual cAMP accumulation data points were entered into a spreadsheet on GraphPad Prism v3.0 (San Diego, CA) and plotted as mean $\pm$ s.e. mean. The data points were fitted to a sigmoidal dose response curve with variable slope by using the built-in non-linear analysis tool. Statistical differences between control and DFO group data points were calculated using a two-tailed Student's *t* test.

Results

Upregulation of A_{2A}R in the striatum of ID mice

As a first step in exploring the potential interaction between iron and the adenosinergic system, we examined the effects of ID on A_{2A}R in the striatum of ID mice. Whereas the ID mice had mean hemoglobin of 7.7 $\pm$ 3.5 g/L, control mice had a value of 15.5 $\pm$ 0.78 g/L. There was no significant difference in the body weights of mice on the control and ID diets (control mice, 25 $\pm$ 1.2 g; ID mice 23 $\pm$ 3.2 g). Immunoblot analysis of striatal tissue samples showed that ID mice had 71% more A_{2A}R protein and a 56% decrease in D2 receptor (Fig. 1). Transferrin receptor (TfR) levels are very tightly regulated by cellular iron status and thus are widely used as cellular markers of iron levels (Lok and Loh, 1998). TfR has been shown to be upregulated in the brains of iron-deficient rats (Han et al., 2003). We measured striatal TfR protein levels as a marker of striatal iron deficiency and found that TfR density was increased by 300% in ID mice compared to control mice, indicating that striatal iron deficiency had been achieved with the low iron diet.

Inbred mouse strains that differ in their striatal iron levels have different levels of striatal A_{2A}R

Since the dietary ID-rodent model also has significant non-CNS, ID-related changes (e.g., anemia) as well as CNS, ID-related changes, we investigated whether the changes in A_{2A}R density in the ID-rodent model were primarily related to changes in brain iron. To achieve this, we selected two derived strains of recombinant inbred mice (strains 40 and 1) that had natural variations in striatal iron (12 $\pm$ 4.4 and 22 $\pm$ 8.6 μ g/g respectively) but had normal peripheral iron stores and hemoglobin (strain 40, 14.22 $\pm$ 0.70 g/dl; strain 1, 10.59 $\pm$ 0.70 g/dl). Immunoblot analysis of the striatum of strain 40 (low iron) showed a 74% increase in A_{2A}R levels as compared to strain 1 (high iron) (Fig. 2). These results indicate that the increased level of A_{2A}R seen in ID mice was most likely not secondary to ID-induced systemic anemia. In fact, the opposite was found: under normal dietary conditions strain 1 mice had both lower hemoglobin and a lower striatal A_{2A}R compared to strain 40 mice.

Upregulation of A_{2A}R and TfR in response to iron chelation in human neuroblastoma cells

In order to explore in more detail the effects of diminished iron on A_{2A}R, we next examined A_{2A}R in cultured SH-SY5Y cells. Expression of endogenous A_{2A}R and A₁R in SH-SY5Y cells was previously demonstrated by radioligand-binding experiments, RT-PCR and Western blots (Salim et al., 2000; and Canals et al., 2005). Cell surface expression for A_{2A}R in neuroblastoma cells was shown in earlier studies (Canals et al., 2005). DFO has been shown to chelate extra- and intracellular iron with a parallel two to threefold increase in TfR after a 24-h incubation

(Bridges and Cudkowicz, 1984). Treatment of SH-SY5Y cells with DFO for 24 h resulted in a significant increase in levels of A_{2A}R; this effect was concentration-dependent with DFO concentrations from 25 to 100 μ M (Figs. 3A, B), though no changes were seen in A₁R protein levels. Studies have shown that heat shock proteins (HSPs), a tightly regulated group of proteins acting as molecular chaperones critical for the maintenance of cellular homeostasis, bind to denatured and malformed proteins and participate in the removal of the wasted proteins (Fink, 1999). Among several multi-member families of HSPs, HSP-70 is one of the best characterized endogenous factors involved in protecting cells from injury under various pathological conditions. Induction or expression of HSPs has been associated with the presence of stressful stimuli (Mosser et al., 1997). Treatment of cells with DFO did not induce the expression of HSP-70, indicating that DFO treatment to these cells does not result in stressful conditions (Fig. 3A). To further rule out the presence of any cell stress condition with DFO treatment we measured the levels of stress-activated kinases (p-SAPK/JNK) which have been shown to be activated in these cells under various stressful conditions (Ferrer et al., 2001). DFO treatment did not result in activation of stressful kinases (Fig. 3A).

Immunocytochemistry results showed a significant increase in the cell surface amounts of A_{2A}R in DFO-treated cells (Figs. 3C, D). DFO also induced an upregulation of TfR indicative of a low cellular iron status. Treatment with 25 μ M DFO for 24 h resulted in a significant increase in TfR density with further increase with 50 μ M DFO (Figs. 3A, B). Time course analysis showed that treatment with 50 μ M DFO resulted in significant increases in the levels of A_{2A}R and TfR at 12 h (Figs. 4A, B). Correlation analysis was performed for the receptor density levels between A_{2A}R and TfR on the pooled results obtained from experiments involving the treatment of cells with 50 μ M DFO. This analysis showed a significant correlation ($r^2=0.79$, $df=22$, $p<0.001$; Fig. 4C).

To investigate the role of transcriptional regulation in the DFO-mediated upregulation of the A_{2A}R, real-time PCR for the A_{2A}R was performed upon cells treated with increasing levels of DFO (10, 25, 50, and 100 μ M) for 24 h. Although results showed a trend for dose-dependent increase in the levels of A_{2A}R message in response to the 24-h DFO treatment, these results were not significant (Fig. 4D).

Iron chelation increases the sensitivity of human neuroblastoma cells to A_{2A}R agonist-induced cyclic AMP production

To investigate the effects of acute iron chelation on the functional capacity of A_{2A}R, we assessed the effect of DFO treatment on the concentration-dependent ability of the selective A_{2A}R agonist, CGS-21680, to increase intracellular cAMP levels. SH-SY5Y cells were either left untreated or treated with DFO for 16 h and then concentration-response (dose response) curves with CGS-21680 (0.1 to 100 nM) were performed. Analysis of the EC₅₀ values from each log concentration response curve after prior treatment with DFO ($n=3$) showed that the mean EC₅₀ of CGS-21680 (0.55 ± 0.29 nM) was significantly lower ($p=0.035$: un-paired two tailed Student's *t* test: GraphPad Prism, San Diego, CA, USA) than the mean EC₅₀ from individual CGS-21680 log concentration response curves in the absence of DFO pre-treatment (4.83 ± 1.33 nM). In addition to this significant potentiation of CGS-21680 activity at low concentrations, there was a small but nonsignificant elevation in the maximal response to CGS-21680 with DFO pre-treatment compared to untreated (Fig. 5). Therefore it appears that with the elevated levels of A_{2A}R there is a commensurate increase in the functional sensitivity of the cells to adenosine agonists in the presence of iron chelation.

Discussion

In this manuscript we have demonstrated that both in cellular systems (SH-SY5Y) and in animal models (ID induced by dietary iron deficiency and inbred strains 1 and 40), low iron

availability results in a significant elevation in A_{2A}R protein levels. In addition to showing an elevation of the protein, we also demonstrated that there is a significant increase in the sensitivity of cells to the effects of A_{2A}R stimulation in a low iron environment. To our knowledge this is the first demonstration that iron chelation (with DFO) induces an increase in A_{2A}R protein levels and functionality. Given the well documented functional interaction of adenosinergic and dopaminergic receptor systems in the striatum (see Introduction), it is likely therefore that such a response to low iron could affect the pathology and etiology of RLS.

We have demonstrated by both in vivo and in vitro approaches that not only does iron insufficiency increase functional A_{2A}R activity, but that this can occur with small decrements in iron concentration. Dietary ID induced a pronounced upregulation of striatal A_{2A}Rs in mice. Moreover, results obtained from inbred mouse strains that differ in their striatal iron levels indicate that this A_{2A}R upregulation results from decreased brain iron content *per se* and not from anemia. Experiments performed in the human neuroblastoma SH-SY5Y cell line indicate that iron regulates the density of A_{2A}R at the cellular level and, also, that there appears to be an intimate physiological relation between cellular iron status and A_{2A}R density as demonstrated by the strong correlation with TfR density.

Adenosine is an endogenous metabolite released by a wide variety of cells when their metabolic status is compromised, especially when there is a mismatch between metabolic activity and oxygen supply (Stone et al., 2007). Iron insufficiency could exert a metabolic deficit in cells with substantial energy demand (Yoon et al., 2003). This local cellular stress could also induce an increase in adenosine release which could be involved in the iron insufficiency-induced A_{2A}R upregulation. Indeed A_{2A}R is upregulated under different stressful conditions (Kobayashi and Millhorn, 1999; Sitkovsky, 2003; Cunha et al., 2006; and Yu et al., 2008). Nonetheless, we did not find significant changes in the density of the proteins HSP-70 and p-SAPK/JNK, which are common markers of cellular stress. Furthermore, A₁R is the main adenosine receptor subtype involved in the neuroprotective effects of adenosine (Dunwiddie and Masino, 2001); (Chen et al., 2007) and no changes were found in A₁R receptor density in cells with diminished iron levels.

The largest body of studies has examined the effects of ID on the dopaminergic system. ID has been shown to decrease D₂R and DAT and increase DA release (Nelson et al., 1997; Erikson et al., 2000; and Erikson et al., 2001). In the present study we confirmed the D₂R downregulation in iron-deficient mice. One connection between iron and the dopamine system is that iron is a co-factor for tyrosine hydroxylase, the rate-limiting enzyme for dopamine production (Cooper et al., 2003). ID is associated with an increase in dopamine release (Nelson et al., 1997), not decrease, however, as would be expected if reduced iron led to reduced dopamine synthesis. In the only study that explored the potential cellular bases of these changes, ID was found to decrease DAT in PC12 cells, by modifications on protein kinase C activity rather than changes in protein synthesis. (Wiesinger et al., 2007). Except for this one study, little else is understood about the iron-dopamine connection. In light of this unknown mechanism for the iron-dopamine connection and because of the known interplay between adenosinergic and dopaminergic system in the striatum (Fuxe et al., 2007), particularly at the level of receptor heterodimer formation (Ferre et al., 2007; and Schiffmann et al., 2007), we examined the effects of ID on the A_{2A}R. Since SH-SY5Y cells do not express D₂R or DAT (Salim et al., 2000) the A_{2A}R upregulation induced by iron insufficiency occurs in these cells and therefore cannot be dependent of D₂R or DAT function. It remains for future studies to determine whether D₂R downregulation is secondary to A_{2A}R upregulation.

Given the strong negative correlation between cellular iron concentrations and A_{2A}R density, the studies broaden the role for iron in adenosine-mediated functions (e.g., motor activity,

sleep, homeostasis and others). And *vice versa*, the findings open up a role for the adenosinergic system in conditions associated with diminished brain iron such as RLS.

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Fig. 1. Dietary iron deficiency increases levels of A_{2A}R and TfR and decreases D₂R in the striatum of mice. A, Representative Western blots from two controls (C, C) and two ID mice striata. Global protein density is controlled with actin immunoblotting B. Densitometric analysis of the results of panel A; data are given as means±SEM (*n*=8 per group). a, *p*<0.001.

Fig. 2. Effects of genetic variability in striatal iron concentration on $A_{2A}R$ levels. (A) Representative Western blots from striata of two strain-40 and two strain-1 animals probed for $A_{2A}R$ and actin. (B) Densitometric analysis (means $\pm$ SEM, $n=8$ per group). a, $p<0.001$.

Fig. 3.

Iron chelation increases levels of A_{2A}R and TfR in cultured human neuroblastoma cells. (A) Representative Western blots of cells treated with 0-100 μM DFO for 24 h. (B) DFO (25-100 μM) concentrations significantly increased both the A_{2A}R and TfR levels after a 24-h exposure; no changes were seen in the levels of A₁R, HSP-70 or SAPK/JNK. Values are mean ± SEM, with 4 animals per group. a, *p* < 0.001 as compared to 0 μM DFO. (C) Representative confocal images of non-permeabilized SH-SY5Y cells, treated with 50 μM DFO for 24 h. Cells were stained with the antibody, which only recognizes the extracellular domain of A_{2A}R, (D) Mean fluorescence intensity ± SEM (*n* = 40 cells) treated with DFO.

Fig. 4.

Time course analysis of the effects of iron chelation on A_{2A}R and TfR levels in human neuroblastoma cells. Cells were treated with 50 μ M DFO for 2, 6, 12, or 24 h. (A) Representative Western blots for A_{2A}R and TfR density with protein control blotting for actin (a, $p < 0.001$). (B) Results from densitometric analysis are shown as means $\pm$ SEM. ($n = 4$ per group, a, $p < 0.001$ (as compared with 0 μ M DFO)). (C) Correlation analysis between A_{2A}R and TfR in cells treated with 50 μ M DFO for 24 h. (D) DFO treatment induced a trend for alterations in A_{2A}R mRNA levels but results were not statistically significant (mean $\pm$ SEM, $n = 6$).

Fig. 5.

Acute iron chelation sensitizes human neuroblastoma cells to selective A_{2A}R ligands. Confluent SH-SY5Y cells were serum-deprived in the presence of adenosine deaminase (2 IU/ml) for 16 h before experimentation. At the same time cells were either treated with a vehicle control or with 50 μ M DFO for 16 h before experimentation. CGS-21680 was applied at each dose specified for 10 min, and cells were then permeabilized, and intracellular cAMP was extracted and assessed by fluorescent immunoassay. Control concentration response curve (black squares) and DFO-treated (open triangles) values represent the mean $\pm$ SEM from three separate experiments. The curve fit was generated using Graph Pad Prism (sigmoidal concentration-response relationship) analysis of the mean responses to three individual experiments comparing CGS-21680 concentration-response series in the presence or absence of DFO pre-treatment.