


# Adenosine A<sub>2B</sub> receptor activation stimulates glucose uptake in the mouse forebrain

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**Abstract** ATP consumption during intense neuronal activity leads to peaks of both extracellular adenosine levels and increased glucose uptake in the brain. Here, we investigated the hypothesis that the activation of the low-affinity adenosine receptor, the A<sub>2B</sub> receptor (A<sub>2B</sub>R), promotes glucose uptake in neurons and astrocytes, thereby linking brain activity with energy metabolism. To this end, we mapped the spatiotemporal accumulation of the fluorescent-labelled deoxyglucose, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), in superfused acute hippocampal slices of C57Bl/6j mice. Bath application of the A<sub>2B</sub>R agonist BAY606583 (300 nM) triggered an immediate and stable (>10 min) increase of the velocity of 2-NBDG accumulation throughout hippocampal slices. This was abolished with the pretreatment with the selective A<sub>2B</sub>R antagonist, MRS1754 (200 nM), and was also absent in A<sub>2B</sub>R null-mutant mice. In mouse primary astrocytic or neuronal cultures, BAY606583

similarly increased <sup>3</sup>H-deoxyglucose uptake in the following 20 min incubation period, which was again abolished by a pretreatment with MRS1754. Finally, incubation of hippocampal, frontocortical, or striatal slices of C57Bl/6j mice at 37 °C, with either MRS1754 (200 nM) or adenosine deaminase (3 U/mL) significantly reduced glucose uptake. Furthermore, A<sub>2B</sub>R blockade diminished newly synthesized glycogen content and at least in the striatum, increased lactate release. In conclusion, we report here that A<sub>2B</sub>R activation is associated with an instant and tonic increase of glucose transport into neurons and astrocytes in the mouse brain. These prompt further investigations to evaluate the clinical potential of this novel glucoregulator mechanism.

**Keywords** Glucose uptake · Adenosine A<sub>2B</sub> receptor · Hippocampus · Striatum · Frontal cortex · 2-NBDG · Lactate · Glycogen

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## Abbreviations

A <sub>2B</sub> R(s)	Adenosine A <sub>2B</sub> receptor(s)
2-NBDG	2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
A <sub>1</sub> R	Adenosine A <sub>1</sub> receptor
FELASA	Federation for Laboratory Animal Science Associations
KO	Knockout
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DMEM	Dulbecco's modified Eagle's medium
HDG	<sup>3</sup> H-2-deoxyglucose
CyB	Cytochalasin B
GLUT1	Glucose transporter type 1

SEM	Standard error of the mean
ANOVA	Analysis of variance
ADA	Adenosine deaminase
DL-TBOA	DL-threo- $\beta$ -Benzoyloxyaspartic acid
GABA	$\gamma$ -aminobutyric acid

## Introduction

The energy homeostasis of the brain is tightly dependent on energetic glucose metabolism occurring either through anaerobic or oxidative pathways [1, 2]. The glucose-derived ATP is consumed by glutamatergic signalling and by the associated activity of  $\text{Na}^+/\text{K}^+$ -ATPases (NKAs) in neurons and astrocytes [3]. Increased neuronal activity thus triggers a physiological accumulation of extracellular adenosine fed by ATP release [4] and ATP consumption by  $\text{Na}^+/\text{K}^+$ -ATPases [5]. Hence, when the circuitry is under heavy load and oxidizes more glucose, peaks of extracellular adenosine may serve as a paracrine and/or autocrine adaptive signal to stimulate glucose metabolism via activating one of its membrane-bound metabotropic receptors [6]. Additionally, pathophysiological conditions, such as transient cerebral ischemia, were shown to exacerbate ATP conversion into adenosine [7, 8]. In fact, the assumption has been toyed with for decades that adenosine generated under energetic crisis protects from cellular damage by restoring energy balance in the brain [9–11].

Accordingly, both of the two most abundant adenosine receptors in the brain, the  $A_1$  and  $A_{2A}$  subtypes [12], have been documented to impact on cerebral glucose metabolism besides being important modulators of neuronal activity [13, 14]. There are two additional cloned adenosine receptors, the  $A_{2B}$  and the  $A_3$  receptors [12], but their roles have been less explored in the brain. Notably,  $A_{2B}R$  have been implicated in peripheral glucose homeostasis [15, 16] and have been proposed to control astrocytic glycogen metabolism [17, 18]. Furthermore, we recently observed that  $A_{2B}R$ s control  $A_1$  receptor ( $A_1R$ )-mediated responses in hippocampal glutamatergic synapses [19]. These observations prompt the attractive hypothesis that  $A_{2B}R$  may be associated with cerebral glucose regulation, especially when a metabolic boost is needed. The therapeutic potential of such (patho)physiological role would be vast.

Here, we measured spatiotemporal and/or quantitative changes in the uptake of different glucose analogues, the synthesis of glycogen and the release of lactate, in frontocortical, hippocampal, and striatal slices, as well as in primary neuronal and astrocytic cultures of C57Bl/6 mice. Overall, we concluded that  $A_{2B}R$ s regulate glucose metabolism in the mouse brain.

## Materials and methods

### Animals

All studies were carried out in accordance with the EU (2010/63/EU) and FELASA guidelines, and they were approved by the Ethical Committee of the Center for Neuroscience and Cell Biology. A total of 50 animals (29 males and 15 dams) were used in the experiments described here. We used 8–10-week-old male  $A_{2B}R$  null-mutant (knock-out, KO) mice with a C57Bl/6 background [20] kindly donated by Drs. Akio Ohta and Michael Sitkovsky (New England Inflammation and Tissue Protection Institute, Northeastern University, Boston, MA, USA) and wild-type C57Bl/6 mice, as well as E17-C57Bl/6 mouse embryos. The adult animals were group housed under controlled temperature ( $23 \pm 2$  °C) and subjected to a fixed 12-h light/dark cycle, with free access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort. The animals used to perform the in vitro studies were deeply anesthetized with halothane before sacrifice (no reaction to handling or tail pinching while still breathing).

### Real-time fluorescent measurement of deoxyglucose uptake in hippocampal slices

Mice were killed, and their brains were quickly removed and placed in ice-cold Krebs-N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) solution (in mM NaCl 113, KCl 3,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4$  1.2,  $\text{CaCl}_2$  2.5,  $\text{NaHCO}_3$  25, glucose 5.5, HEPES 1.5, and pH 7.4). Coronal slices (300  $\mu\text{m}$  thickness) were cut with a Vibratome 1500 (Leica, Germany), left to recover for 1 h at room temperature in Krebs-HEPES solution gassed with 5 %  $\text{CO}_2$  and 95 %  $\text{O}_2$ , then gently mounted on coverslips with the hippocampus in the center, and placed in a RC-20 superfusion chamber on a PH3 platform (Warner Instruments, Harvard, UK). The slices were superfused with gassed Krebs-HEPES solution at a rate of 0.5 mL/min in a closed circuit, and they were then photographed with a CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA) every 30 s over the following 30 min, using a  $5\times$  Plan Neofluar objective (NA 0.25) on an inverted Axiovert 200-M fluorescence microscope (Carl Zeiss, Germany), coupled to a Lambda DG-4-integrated 175-W light source and wavelength switching excitation system (Sutter Instrument Company, Novato, CA, USA) to allow real-time video imaging. The data were band-pass filtered for excitation (470/40) and emission (525/50).

After recording six images for autofluorescence (to establish the baseline), 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (30  $\mu\text{M}$ ) was applied through the reservoir of the closed superfusion circuit. As within 10 min, the increase of 2-NBDG signal reached linearity

(Supplementary Fig. 1), we recorded a 10-min predrug period following the first 10 min of 2-NBDG application. Subsequently, at 20 min, the slices were challenged with the A<sub>2B</sub>R agonist BAY606583 (300 nM) or its vehicle, dimethyl sulfoxide (DMSO) (0.1 % v/v), and another 10-min period was recorded at every 30 s. To antagonize A<sub>2B</sub>Rs, MRS1754 (200 nM) was applied 3 min before 2-NBDG. All measurements were obtained in duplicate from each animal (see [Supplementary Material](#) for additional details and calculation).

### Cell culture preparation

Neuronal and astrocytic primary cultures were prepared as described elsewhere [21, 22]. The neocortex or hippocampi from E17 male and female C57Bl/6 mouse embryos were digested for 15 min with 0.125 % trypsin (type II-S, from porcine pancreas, Sigma-Aldrich, Sintra Portugal) and 50 µg/mL deoxyribonuclease (DNase) (Sigma-Aldrich) in Hank's balanced salt solution without calcium and magnesium (in mM NaCl 137, KCl 5.36, KH<sub>2</sub>PO<sub>4</sub> 0.44, NaHCO<sub>3</sub> 4.16, Na<sub>2</sub>HPO<sub>4</sub> 0.34, D-glucose 5, and pH 7.2). After dissociation, astrocytes were grown in plastic Petri dishes for 14 days in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin (Sigma-Aldrich), at 37 °C in an atmosphere of 95 %/5 % air/CO<sub>2</sub>, replacing half of the medium after 7 days. The cells were then trypsinized and plated onto poly-D-lysine- (100 µg/mL, Sigma-Aldrich) and laminin-coated (10 µg/mL, Sigma-Aldrich) 24-well culture plates at a cell density of 150,000/cm<sup>2</sup>. Uptake assays were performed 24 h later. For neuronal cultures, cells were plated directly onto poly-D-lysine- and laminin-coated 24-well culture plates, at the same cell density, in DMEM plus 10 % fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Two hours after seeding, the medium was replaced by Neurobasal medium with 2 % B27 supplement (GIBCO, Life Technologies), 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM glutamine (Sigma-Aldrich), and cells were grown at 37 °C in an atmosphere of 95 %/5 % air/CO<sub>2</sub> until they were used 14 days later. The medium was partially (40 %) replaced every 4 days.

### <sup>3</sup>H-deoxyglucose uptake in neuron and astrocyte cultures

We used six different cerebrocortical astrocyte cultures, as well as five cerebrocortical and four hippocampal neuronal cultures, obtained from E17 mouse embryos of 11 pregnant dams, respectively. After 15 days in culture, the culture medium was replaced with 250-µL Krebs-HEPES assay solution (pH 7.4; 37 °C) (see above), containing either the vehicle alone (0.1 % DMSO, in 14 wells of the 24-well plate), or the A<sub>2B</sub>R antagonist, MRS1754 (200 nM, in the rest of the plate). The cultures were incubated for 60 min at 37 °C, and

subsequently, the glucose transporter blocker, cytochalasin B (final concentration, 10 µM; 4 wells/plate) or BAY606583 (final concentration, 300 nM; in five DMSO-pretreated wells), or BAY606583 in combination with MRS1754 (in five MRS1754-pretreated wells), or the vehicle for BAY606583 (DMSO) alone (5 wells) or in combination with MRS1754 (the last 5 wells) were gently added in a volume of 250 µL, together with the radioactive glucose analogue <sup>3</sup>H-2-deoxyglucose (<sup>3</sup>HDG; 16.6 nM, final concentration). After a further 20-min incubation at 37 °C, the plates were transferred to ice and aliquot of 182 µL was taken from each well to determine the exact <sup>3</sup>HDG concentration. The cells were then washed gently three times with 1-mL ice-cold Krebs-HEPES and were subsequently dissolved with 300 µL NaOH (0.5 M) on a shaker for 1 h to recover the proteins and the <sup>3</sup>HDG taken up by the cells. The tritium content of the samples was measured in a Tricarb β-counter (PerkinElmer, Portugal, ILC Ltd., Lisbon), and these values were adjusted for protein quantity and the <sup>3</sup>H concentrations in each individual well and multiplied by 3.3 × 10<sup>5</sup> (the ratio between <sup>3</sup>HDG and D-glucose in the medium).

We validated this protocol by measuring the glucose uptake in the presence of cytochalasin B (CyB; 10 µM), which inhibits some glucose transporters, including glucose transporter type 1 (GLUT1), in astrocytes [23, 24]. CyB largely inhibited the uptake of glucose in astrocytes (*n*=5, *P*<0.001 vs. DMSO control; Fig. 2), and as expected, CyB had a much smaller but still significant effect on neuronal glucose transport (*n*=4, *P*<0.05; Fig. 3), which indicates a gain-of-function for GLUT1 in neuronal culture [25].

### In vitro <sup>3</sup>H-deoxyglucose uptake in brain slices

To better characterize the endogenous glucoregulator role of A<sub>2B</sub>R in different brain areas typically affected by neuropsychiatric disorders, we moved to an in vitro glucose uptake protocol previously optimized in acute hippocampal slices, which allows simultaneously comparing the effect of various treatments [26]. Mice were anesthetized with halothane, sacrificed (around 2:00 P.M. each experimental day to reduce potential circadian hormonal effects), and their brain was immediately placed in ice-cold Krebs-HEPES assay solution (see above). The pairs of frontal cortices, hippocampi, and striata were rapidly dissected and cut in 400-µm-thick transverse slices with the help of a McIlwain tissue chopper, and the slices were gently separated in ice-cold assay solution (carboxygenated with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>), then transferred and maintained at 37 °C in a multichamber slice incubator with 50 mL of carboxygenated assay solution until the end of the experiment. Each container had separated nylon-mesh bottom wells to keep three cortical or five hippocampal or three striatal slices from each of the three animals per experiment; i.e., each container had approximately 7.2 mg protein

in 50-mL assay medium. MRS1754 (100 nM) or its vehicle, DMSO (0.1 %), or adenosine deaminase (3 U/mL) were bath applied from the beginning of the batch incubation period; that is, the 60 min of recovery period was used for the pretreatment as well. After 60 min of preincubation, the radioactive glucose analogue,  $^3\text{HDG}$  (final concentration of 2 nM), was applied to the bath for 30 min. The slices were washed twice in ice-cold assay solution for 5 min and collected in 1 mL NaOH (0.5 M) to dissolve the slices. An aliquot of 800  $\mu\text{L}$  was then assayed for  $^3\text{H}$  in a Tricarb  $\beta$ -counter, while the remaining solution was used to quantify the protein with a bicinchoninic acid assay (Merck Biosciences, Germany). Tritium uptake was multiplied by a factor of  $2.75 \times 10^6$  to estimate the total glucose uptake (corresponding to the concentration difference between D-glucose and  $^3\text{HDG}$  in the uptake medium [26]).

### In vitro measurement of glycogen accumulation and lactate release in brain slices

The experimental layout was essentially the same as for the  $^3\text{HDG}$  uptake with slight modifications: since MRS1754 (100 nM) strongly reduced  $^3\text{HDG}$  uptake in the bathed slices at 37 °C (Fig. 3), we selected this  $\text{A}_{2\text{B}}\text{R}$  antagonist to modulate glycogen synthesis and lactate release. We used the frontal cortex of eight mice and the striata of six mice for this assay as these brain areas provide more tissue and hence better signal-to-noise ratio. MRS1754 or its vehicle, DMSO, was co-administered in the bath with D-glucose labelled at all the six carbon atoms ( $^{14}\text{C}_6\text{-D-glucose}$ ) at the concentration of 50 nM from the beginning of the 90-min incubation period. This protocol allowed us to monitor the recovery of glycogen stores which become depleted when sacrificing the animals [36]. Glycogen was separated from the tissue as before [26]. Upon completion of the 90-min incubation period, a sample was taken from the bath to determine lactate release. The  $^{14}\text{C}$  concentration in the bath as well as total  $^{14}\text{C}$  retention in the slices and  $^{14}\text{C}$  incorporation in glycogen were determined with the  $\beta$ -counter, while lactate levels were measured with a lactate dehydrogenase kit (Sigma-Aldrich) and expressed as nanomole per mg of tissue protein.

### Data presentation and statistical analysis

All data are expressed as the means  $\pm$  SEM of the number of independent observations ( $n$ ), each averaged from duplicates, quadruplicates, or quintuplicates as indicated. These data were normalized to the respective DMSO control, and tested for normality with the Kolmogorov-Smirnov normality tests. Statistical significance was calculated by one sample  $t$  test against the hypothetical value of zero or with one-way analysis of variance (ANOVA) with Dunett's post hoc test when

more than two groups were compared and a value of  $P < 0.05$  was accepted as significant.

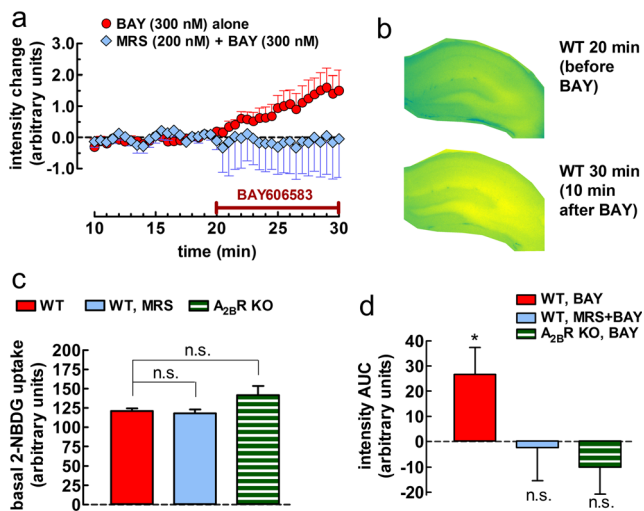
### Chemicals

$^3\text{HDG}$  (specific activity 60 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA).  $^{14}\text{C}_6\text{-D-glucose}$  ( $^{14}\text{C}$ -glucose; specific activity 0.36 Ci/mmol) was bought from PerkinElmer Portugal (ILC Lda., Lisbon, Portugal). 2-NBDG was acquired from Invitrogen (Carlsbad, CA, USA). Non-organic reagents, adenosine deaminase (ADA), cytochalasin B, HEPES, and DMSO were purchased from Sigma-Aldrich Portugal (Sintra, Portugal) or Merck Biosciences (Darmstadt, Germany). BAY606583 and MRS1754 were obtained from Tocris Bioscience (Bristol, UK). All non-water soluble substances and 2-NBDG were reconstituted in DMSO at 1000 $\times$  the final concentration and stored in aliquots at  $-20$  °C.

### Results

#### $\text{A}_{2\text{B}}\text{R}$ activation rapidly enhances glucose uptake in hippocampal slices

First, we assessed whether  $\text{A}_{2\text{B}}\text{R}$  activation affects glucose uptake in superfused hippocampal slices by monitoring the rate of fluorescence-labelled deoxyglucose (2-NBDG) accumulation. The basal accumulation of 2-NBDG was most evident in astrocyte-rich areas of transverse hippocampal slices, i.e., in the *stratum lacunosum*, followed by the *strata radiatum* and *moleculare*, and the smallest signal was found in the *strata pyramidale* and *granulare* (Fig. 1a, b), consistent with previous findings [27]. The pharmacological activation of  $\text{A}_{2\text{B}}\text{R}$  with BAY606583 (300 nM, i.e., at a concentration selective for this receptor) rapidly increased the velocity of 2-NBDG accumulation in these hippocampal slices prepared from the wild-type (WT) mice ( $n=7$  in duplicate,  $P < 0.05$ ), as gauged from the fluorescence intensity in the whole surface of the transversal slice (Fig. 1b) and compared to the average response in 2-NBDG accumulation in the pair of DMSO-treated slices (Fig. 1a). Pretreatment of the slices with the selective  $\text{A}_{2\text{B}}\text{R}$  antagonist MRS1754 (200 nM) for 23 min prevented BAY606583 from stimulating 2-NBDG uptake ( $n=4$ ,  $P > 0.05$ ; Fig. 1e). Additionally, BAY606583 also failed to stimulate 2-NBDG uptake in the hippocampal slices prepared from  $\text{A}_{2\text{B}}\text{R}$  knockout (KO) mice ( $n=6$ ,  $P > 0.05$ ; Fig. 1e). Importantly, 2-NBDG accumulation was not statistically different from the WT control neither after pretreatment with MRS1754 ( $n=4$ ,  $P > 0.05$ ; Fig. 1d) nor in  $\text{A}_{2\text{B}}\text{R}$  KO mice ( $n=6$ ,  $P > 0.05$ ; Fig. 1d), even though the uptake velocity was tendentially greater in  $\text{A}_{2\text{B}}\text{R}$  KO mice ( $117.3 \pm 9.8$  % of WT



**Fig. 1** Time course and regional variation of the effect of  $A_{2B}R$  activation on the uptake of the fluorescent glucose analogue, 2-NBDG, in 300- $\mu$ m-thick transversal hippocampal slices of young C57Bl/6j male mice. **a** The  $A_{2B}R$  agonist BAY606583 (BAY; 300 nM) rapidly increased the accumulation of 2-NBDG throughout the hippocampal slices of the wild-type (WT) mice. The  $x$  axis denotes the time of exposure to 2-NBDG (30  $\mu$ M) and indicates that BAY606583 was applied 20 min after the addition of the tracer 2-NBDG. The  $y$  axis indicates that the values on the graph are the mean  $\pm$  SEM of net changes ( $n=7$  for BAY606583 alone and  $n=4$  for BAY606583+MRS1754 (200 nM, an  $A_{2B}R$  antagonist)), after the subtraction of the mean intensity values of the respective DMSO controls in each experiment. **b** Representative images taken from a WT hippocampal slice immediately before and 10 min after the beginning of BAY606583 superfusion. The false color scale illustrating the distribution of the fluorescence signal ranges from *blue* (low signal) through *green* (moderate signal), up to *yellow* (strong signal). **c** The bar graph summarizes the rate of basal 2-NBDG accumulation, expressed in arbitrary intensity units, under control condition (left, red bar;  $n=7$ ) or after  $\sim$ 23 min of preincubation with MRS1754 (middle, light blue bar;  $n=4$ ) in slices from WT mice, and under control condition in slices from  $A_{2B}R$  KO ( $-/-$ ) mice (right, striped green bars;  $n=6$ ). Each bar represents the mean  $\pm$  SEM of signal intensities in quadruplicates in four to seven animals at the linear phase, i.e., 19.5 min after the addition of 2-NBDG; *n.s.* not significant, as assessed with one-way ANOVA followed by Bonferroni's multiple comparison's post hoc test. **d** Effect of drugs normalized to the respective DMSO controls (dashed line crossing the zero value). Values represent the mean  $\pm$  SEM of the individual measurements in duplicates, as obtained with the area under the curve (AUC) method for BAY606583 versus DMSO application in the absence ( $n=7$ ) or in the presence of MRS1754 ( $n=4$ ) in the WT mice, as well as in the absence of  $A_{2B}R$  ( $A_{2B}R$  KO;  $n=6$ ).  $*P<0.05$ . See [Supplementary Material](#) for additional details and calculation

control,  $P>0.05$ ). See [Supplementary Material](#) for additional details and calculation.

### $A_{2B}R$ activation stimulates glucose transport in cultured astrocytes and neurons

Since the uptake of 2-NBDG underrepresents neuronal glucose transport [27], we next measured the effect of acute *in vitro*  $A_{2B}R$  activation on glucose uptake in primary mouse astrocytes and neurons, using a  $^3H$ DG transport assay.

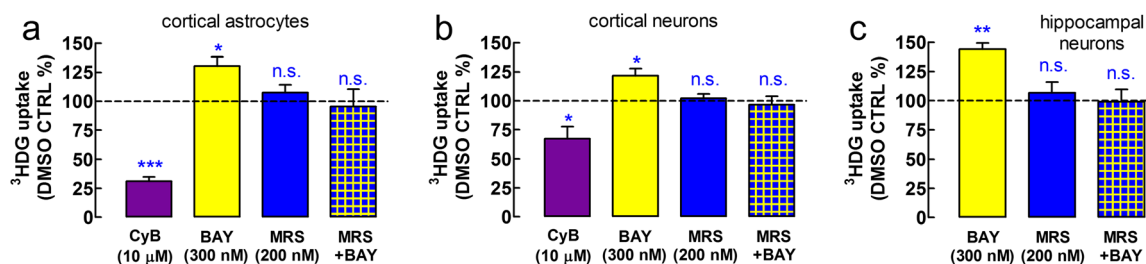
Glucose uptake during the 20-min assay amounted to  $36.2 \pm 5.8$  nmol/mg protein in astrocytes ( $n=6$  in quadruplicates),  $25.9 \pm 6.6$  nmol/mg protein in the cerebrocortical neurons ( $n=5$ ), and  $10.3 \pm 5.5$  nmol/mg protein ( $n=4$  in triplicates) in the hippocampal neurons. BAY606583 (300 nM) stimulated the uptake of glucose by  $30.4 \pm 7.9$  % ( $n=6$ ;  $P<0.05$  vs. DMSO control) in astrocytes (Fig. 2a), by  $21.8 \pm 5.9$  % ( $n=5$ ;  $P<0.05$ ) in cerebrocortical neurons (Fig. 2b), and by  $43.9 \pm 10.7$  % ( $n=4$ ;  $P<0.01$  vs. DMSO control) in hippocampal neurons (Fig. 2c). The pretreatment with the  $A_{2B}R$  antagonist, MRS1754 (200 nM), did not affect the uptake of glucose indicating a lack of endogenous tone in the cultured cells (Fig. 2). However, MRS1754 prevented BAY606583 from stimulating glucose uptake in both cell types (Fig. 2), indicating the involvement of  $A_{2B}R$ .

### The inhibition of endogenous $A_{2B}R$ activity strongly decreases glucose uptake in brain slices

Next, we asked if  $A_{2B}R$ s can also function as an endogenous glucoregulator in different brain areas. To this end, we batch-incubated acute frontocortical, hippocampal, or striatal slices from C57Bl/6 mice at 37  $^{\circ}C$  under continuous oxygenation. The control resting glucose uptake in the subsequent 30-min period was not different among the three brain regions investigated ( $n=11-14$ ,  $P>0.05$ ) (Fig. 3). We previously saw in rat hippocampal slices that the glutamate reuptake inhibitor, DL-TBOA (10  $\mu$ M), significantly reduces resting  $^3H$ DG uptake by 23 % in a similar assay (unpublished data), indicating that glutamate recycling is responsible for almost one fourth of resting glucose uptake via the stimulation of  $Na^+/K^+$ -ATPases [28]. This is expected to contribute to extracellular adenosine accumulation. Indeed, 1-h pretreatment and 30-min treatment with MRS1754 (100 nM) reduced glucose uptake in the three brain areas by  $\sim$ 33–41 % ( $n=6$ ,  $P<0.01$ ) as compared to control (Fig. 3). This suggest that extracellular adenosine accumulation in these bathed slices at 37  $^{\circ}C$  is sufficient to activate  $A_{2B}R$ s. Certainly, the reduction of extracellular adenosine levels with 1-h pretreatment and 30-min treatment with adenosine deaminase (3 U/mL) similarly diminished glucose uptake by 17–36 % in slices from the three brain areas ( $n=5-6$ ,  $P<0.05$ ) (Fig. 3).

### The effect of $A_{2B}R$ blockade on glycogen synthesis and lactate release

Glycogen levels and lactate release are major parameters reflecting the modulation of glucose metabolism [28, 29]. Hence, with the help of the metabolizable glucose analogue  $^{14}C$ -glucose, we measured total glucose uptake and  $^{14}C$  incorporation into glycogen in frontocortical and striatal slices in the 90-min recovery period. Total glucose  $^{14}C$  retention by the striatal slices during the 90-min recovery period ( $78.8 \pm$

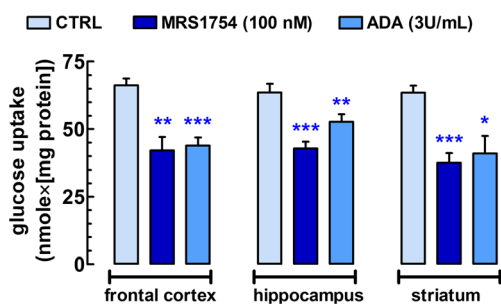


**Fig. 2**  $A_{2B}R$  activation stimulates glucose uptake in cerebrocortical astrocytes as well as in cerebrocortical and hippocampal neurons *in vitro*. The  $A_{2B}R$ -selective agonist BAY606583 (BAY; 300 nM;  $n=6$ ) stimulated glucose uptake in primary cultures of **a** cerebrocortical astrocytes, **b** cerebrocortical neurons, and **c** hippocampal neurons, obtained from E17-C57Bl/6 mouse embryos, as assessed using the 20-min <sup>3</sup>H-DG uptake assay. One-hour pretreatment with the  $A_{2B}R$ -selective antagonist, MRS1754 (MRS; 200 nM;  $n=5$ , astrocytes;  $n=4$ , neurons), prevented the action of the  $A_{2B}R$  agonist, while it had no effect on uptake *per se* ( $n=5$ , astrocytes;  $n=4$ , neurons). D-glucose uptake is calculated according to [21] and is expressed in nanomole per milligram protein, mean±SEM of “ $n$ ” cultures in quadruplicates for the cerebrocortical

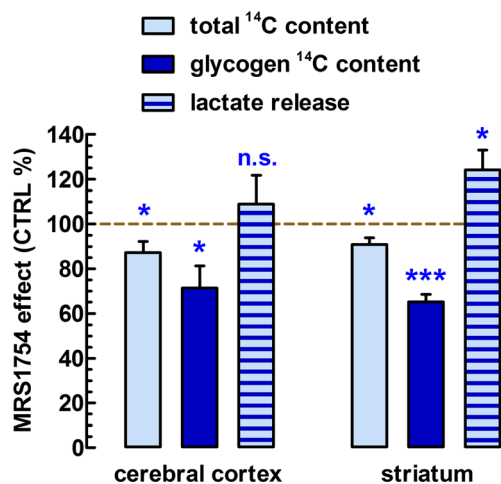
cultures (4 wells/treatment/culture) and triplicates for the hippocampal cultures. Cytochalasin B (CyB; 10 μM) inhibited the uptake of glucose in five astrocytic cultures stronger than in four cerebrocortical neuronal cultures, because neuronal glucose uptake is moderately dependent on GLUT1 transporters in culture, while GLUT1 is the primary glucose transporter in astrocytes [20]. CyB was not tested in hippocampal neurons. After 60-min preincubation at 37 °C in the assay medium, <sup>3</sup>H-DG was co-administered for 20 min with BAY, CyB, or their vehicle, DMSO. When used, MRS1754 was present since the beginning of the preincubation period. \* $P<0.05$ , \*\*\* $P<0.001$  versus DMSO control; *n.s.* not significant

13.2 nmol/mg protein) was significantly smaller (by  $9.2\pm 3.1\%$ ,  $n=6$ ,  $P<0.05$ ) in the presence of MRS1754 (100 nM) (Fig. 4), which was a considerably lesser effect amplitude than what we measured with <sup>3</sup>H-DG after 1-h recovery. Moreover, 20 % of the above total retention value represented <sup>14</sup>C<sub>6</sub>-glucose incorporation into glycogen ( $15.4\pm 3.2$  nmol/mg protein). As expected, MRS1754 also greatly decreased striatal glycogen levels by  $34.9\pm 3.4\%$  ( $P<0.05$ ) (Fig. 4). Last but not least, lactate loss from the control striatal slices to the medium amounted to  $621\pm 78$  nmol/mg protein. MRS1754 significantly stimulated lactate release by  $24.1\pm 8.9\%$  ( $n=6$ ,  $P<0.05$ ) (Fig. 4).

Frontocortical total <sup>14</sup>C retention amounted to  $87.9\pm 9.8$  nmole/mg protein ( $n=8$ ). Glycogen synthesis was  $18.2\pm 4.0$  nmol/mg protein (20.7 % of total <sup>14</sup>C retention), and lactate loss to the bath was  $504\pm 56$  nmol/mg protein ( $n=8$ ); i.e., none of these three parameters were statistically different from



**Fig. 3** Endogenously active  $A_{2B}R$  tonically stimulate glucose uptake in acute 400-μm-thick coronal frontocortical, transversal hippocampal, and rostrocaudal striatal slices from C57Bl/6j mice. Brain slices were divided into four groups, and they were incubated in the assay solution at 37 °C under continuous gassing with a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. One chamber with a group of slices from the three brain areas was exposed to MRS1754, another group was exposed to adenosine deaminase (ADA; 3 U/mL), while the remaining two groups served as control. After 60-min pretreatment, <sup>3</sup>H-DG (2 nM) was bath applied to trace the course of glucose uptake for a 30-min period. Total D-glucose uptake (nmol/mg protein) was determined for each group of slices (for further details, see Materials and Methods and [26]). Bars represent the mean±SEM of individual measurements from  $n=5-14$  mice. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  versus control



**Fig. 4** Mapping the role of  $A_{2B}R$ s on glycogen synthesis and lactate release. In acute 400-μm-thick coronal frontocortical and rostrocaudal striatal slices from C57Bl/6j mice, brain slices were divided into two groups and they were submerged in an assay solution containing the metabolizable glucose analogue, <sup>14</sup>C<sub>6</sub>-glucose (50 nM), at 37 °C under continuous gassing with a mixture of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. One chamber with a group of slices from the three brain areas was exposed to MRS1754; the other group was exposed to its vehicle, DMSO (0.1 %, control). After 90-min incubation, the slices were washed three times in ice-cold assay solution, collected in 2 mL NaOH (0.5 M). After dissolving the slices, 200 μL of these samples was used to count total <sup>14</sup>C retention in the slices and protein quantities; the rest of the 1800 μL was used to precipitate glycogen and count its <sup>14</sup>C content as before [26]. Lactate content in the bath at the end of the 90-min incubation period was assessed with a lactic acid assay kit. Bars represent the mean±SEM of individual measurements from  $n=6-8$  mice. \* $P<0.05$ , \*\*\* $P<0.001$  versus control

those in the striatum ( $P>0.05$ ).  $A_{2B}R$  blockade reduced both  $^{14}C$  retention by  $12.8\pm 4.0\%$  ( $P<0.05$ ) and  $^{14}C$ -labelled glycogen content by  $38.6\pm 9.9\%$  ( $P<0.05$ ) but did not affect lactate loss to the medium ( $P>0.05$ ) (Fig. 4).

## Discussion

The present study provides a direct pharmacological demonstration that the activation of adenosine  $A_{2B}$  receptors ( $A_{2B}R$ ) triggers a rapid and sustained glucose uptake in the mouse brain. This effect appears to occur both in neurons and astrocytes. Hence,  $A_{2B}R$  may link extracellular adenosine peaks with increased glucose uptake, thus allowing energy metabolism to meet the demands of neural activity. Such mechanism would be essential to avoid an hypoenergetic crisis, which might lead to cell death. Indeed,  $A_{2B}R$  activation has been linked with increased survival of hippocampal and cortical cells under oxygen-glucose deprivation [30–32]. Hence,  $A_{2B}Rs$  seem to generally assist the homeostasis of brain cells in a manner similar to that occurring in the periphery.

Thus, it is well-known that  $A_{2B}Rs$  have widespread direct and indirect roles in the control of blood glucose levels and systemic energy metabolism. Indirect mechanisms involve the modulation of peripheral insulin sensitivity and tissue inflammation [15, 16, 33, 34]. Apart from this,  $A_{2B}Rs$  also can directly regulate glucose handling by the three major organs involved in peripheral gluoregulation, i.e., the liver, the white adipose tissue, and the skeletal muscle [35].

In the brain, compelling evidence supports that glycogen is a major glucose storage with a buffer function in astrocytes to mitigate the potential energetic crisis under prolonged intense activity of the circuitry [36, 37]. Importantly, the activation of  $A_{2B}Rs$  can promote Akt phosphorylation [16, 38], which in turn can stimulate glycogen synthesis. Furthermore, lactate production can be partly supported by glycogenolysis in astrocytes, and during activity, neurons and thin astrocytic processes in the synapse mostly rely on the oxidation of lactate rather than that of glucose [1, 37]. We indirectly demonstrated here that  $A_{2B}R$  activation is positively linked with glycogen synthesis and negatively to lactate output in the striatal slice. Overall, these observations prompt the following scenario: during strong activity of the circuitry, high extracellular levels of adenosine are generated, which in turn will stimulate the replenishment of astrocytic glycogen stores, via  $A_{2B}R$  activation. This is probably associated with a dampening effect on lactate release from astrocytes. This can be regarded as a counterregulatory process—a metabolic brake that protects astrocytes from glucose storage depletion and can also reduce extracellular acidification.

There is ample evidence for the presence of  $A_{2B}Rs$  in both neurons and astrocytes throughout the mammalian brain [39]; thus,  $A_{2B}Rs$  are well-positioned to modulate glucose

metabolism in both brain cell types. In our neuronal cultures, glutamatergic cells were the likely candidates to respond to  $A_{2B}R$  activation with increased glucose uptake. It is because hippocampal and cerebrocortical cultures at this age contain predominantly excitatory neurons [40, 41]. The principal neurons of the cerebral cortex and hippocampus are the glutamatergic pyramidal cells. This predicts that  $A_{2B}R$  activation can regulate neuronal besides astrocytic metabolism in those brain areas. Normally, glutamatergic neurons use either glucose or lactate [42], so if  $A_{2B}R$  activation increases glucose use in pyramidal cells, those neurons would concomitantly take up less lactate. Consequently,  $A_{2B}R$  blockade would tip the balance to greater lactate uptake. The simultaneous stimulation of lactate release in astrocytes and lactate oxidation in pyramidal cells should result in no net change in lactate outflow to the medium, in accordance with our observations. In contrast,  $A_{2B}R$  blockade did not affect lactate turnover in the same way in the striatal slices. From the culture data, it is not easy to disentangle if  $A_{2B}R$  activation has any effect on glucose or lactate use in striatal neurons, because these cells are predominantly GABAergic, and there is no evidence for glutamatergic neurons whatsoever [43]. This is a probable reason for the difference in frontocortical and striatal lactate loss to the medium.

Besides the direct regulation of glucose transport, the modulation of glutamatergic signalling will also affect glucose use [1]. This is because glutamatergic neurotransmission is a costly process, being responsible for the majority of energetic glucose metabolism in the brain [3]. In concert with this, we recently found that  $A_{2B}Rs$  modulate glutamatergic synaptic transmission in the hippocampus [19]. Not only neurons but also astrocytes are equipped with  $A_{2B}R$  [39, 44], and both cell types are capable of releasing glutamate [45]. Therefore,  $A_{2B}Rs$  are well-positioned in the tripartite synapse to coordinate glucose uptake according to metabolic demands.

The present pharmacological study is based on the use of a selective  $A_{2B}R$  agonist, BAY606583, and an antagonist of this adenosine receptor, MRS1754, together with  $A_{2B}R$  KO mice. Hence, the lack of effect of BAY606583 either in the presence of MRS1754 or in the absence of  $A_{2B}R$  provides compelling evidence for the involvement of  $A_{2B}R$  in the action of BAY606583. Interestingly, there was a tendency for an increased 2-NBDG influx in the hippocampal slices from  $A_{2B}R$  KO mice, which contradicts what one would expect, i.e., a decreased glucose uptake in the absence of  $A_{2B}R$ . However,  $A_{2B}R$  KO mice exhibit an apparent systemic metabolic dysregulation [34] that may also have an impact on cerebral gluoregulation—an additional confounding factor to be taken into account when the cerebral gluoregulator role of  $A_{2B}R$  is investigated in future studies.

Glucose availability in the brain regulates cognition and memory in both healthy humans and dementia patients [46–48]. Although normally metabolic boosters (nootropics)

do not mitigate the outcome of neurodegenerative disorders, A<sub>2B</sub>R agonists eventually possess multiple beneficial actions including boosting cerebral glucose metabolism and increasing cell survival apart from effects on energy metabolism [30–32]. Therefore, further studies are invited to evaluate the clinical potential of A<sub>2B</sub>R agonists in both animals and man.

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#### Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interests to report and received no financial support or compensation from any individual or corporate entity over the past 3 years for research or professional services, and there are no personal holdings that could be perceived as constituting a potential conflict of interest.

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