RESEARCH PAPER

Role of β-adrenoceptors in glucose uptake in astrocytes using β-adrenoceptor knockout mice

Stephanie L Catus1,2, Marie E Gibbs3, Masaaki Sato1*, Roger J Summers1,2 and Dana S Hutchinson1,2

1Department of Pharmacology, Monash University, Clayton, Victoria, Australia, 2Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia, and 3Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria, Australia

BACKGROUND AND PURPOSE

β1-, β2- and β3-adrenoceptors determined by functional, binding and reverse transcription polymerase chain reaction (RT-PCR) studies are present in chick astrocytes and activation of β2- or β3-adrenoceptors increase glucose uptake. The aims of the present study are to identify which β-adrenoceptor subtypes are present in mouse astrocytes, the signal transduction mechanisms involved and whether β-adrenoceptor stimulation regulates glucose uptake.

EXPERIMENTAL APPROACH

Astrocytes were prepared from four mouse strains: FVB/N, DBA/1 crossed with C57BL/6J, β3-adrenoceptor knockout and β1β3-adrenoceptor knockout mice. RT-PCR and radioligand binding studies were used to determine β-adrenoceptor expression. Glucose uptake and cAMP were assayed to elucidate the signalling pathways involved.

KEY RESULTS

mRNAs for all three β-adrenoceptors were identified in astrocytes from wild-type mice. Radioligand binding studies identified that β1- and β3-adrenoceptors were predominant. cAMP studies showed that β1- and β2-adrenoceptors coupled to Gs whereas β3-adrenoceptors coupled to both Gs and Gi. However, activation of any of the three β-adrenoceptors increased glucose uptake in mouse astrocytes. Interestingly, there was no functional compensation for receptor subtype loss in knockout animals.

CONCLUSIONS AND IMPLICATIONS

This study demonstrates that although β1-adrenoceptors are the predominant β-adrenoceptor in mouse astrocytes and are primarily responsible for cAMP production in response to β-adrenoceptor stimulation, β3-adrenoceptors are also present in mouse astrocytes and activation of β2- and β3-adrenoceptors increases glucose uptake in mouse astrocytes.

Abbreviations

BSA, bovine serum albumin; CGP12177A, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride; CGP20712A, (±)-2-hydroxy-5-(2-((2-hydroxy-3-(4-(1-methyl-4-(trifluoromethyl)-1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethanesulphonate; CL316243 (R,R)-5-[2-[(3-chlorophenyl)-2-hydroxyethyl][amino]-propyl][1,3-benzodioxole-2,2-dicarboxylate; DMEM, Dulbecco’s modified Eagle’s medium; dNTP, deoxyribonucleotide triphosphate; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; ICI118551, erythro-DL-1(7-methylin-dian-4-yloxy)-3-isopropylaminobutan-2-ol; KO, knockout; PBS, phosphate buffered saline; PTX, Pertussis toxin; RT-PCR, reverse transcription polymerase chain reaction; SR59230A, 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propanol oxalate
Introduction

Adrenoceptors are G protein-coupled receptors (GPCRs) comprising three types: α1C, α2A and β-adrenoceptors (nomenclature follows Alexander et al., 2009). α1C-Adrenoceptors couple to Gi to activate phospholipase C and increase inositol trisphosphate and diacylglycerol; α1D-adrenoceptors couple to Gs to activate phospholipase C and increase intracellular cAMP levels; whereas β1-adrenoceptors couple to Gs, to negatively regulate adenyl cyclase and cAMP levels; whereas β2-adrenoceptors couple to Gi to positively regulate adenyl cyclase to increase intracellular cAMP levels (Alexander et al., 2009). While β1-adrenoceptors primarily couple to Gi, coupling to Gs, has also been documented for the β2-adrenoceptor (Nicholas, 2005; Sato et al., 2009) and β3-adrenoceptors (Hutchinson et al., 2002; 2007; Sato et al., 2005; 2007). β1A and β2-adrenoceptors are splice variants of the β1-adrenoceptor (Evans et al., 1999) that show identical pharmacological profiles (Hutchinson et al., 2002), but the β2-adrenoceptor couples solely to Gs whereas the β2D-adrenoceptor couples both to Gs and Gi (Hutchinson et al., 2005; Sato et al., 2005; 2007; 2008).

In rodents, β-adrenoceptors are expressed throughout the whole brain (Nicholas et al., 1993). In many brain regions, β1- and β2-adrenoceptors are expressed at similar densities but β1-adrenoceptors predominate in the cerebellar cortex, hippocampus and basal ganglia (Paschalis et al., 2009) whereas β2-adrenoceptors predominate in the cerebellum and thalamus (Rainbow et al., 1984). β1-Adrenoceptors in the rodent brain are limited to the hippocampus, hypothalamus, amygdala and cerebral cortex (Summers et al., 1995). β1-Adrenoceptors have roles in the regulation of food intake (Tsujii and Bray, 1998) and for the treatment of anxiety and depressive disorders (Stemmell et al., 2008) and activation following intraventricular injection of β1-adrenoceptor agonists increases c-fos expression in the hypothalamus (Castillo-Melendez et al., 2000). The β1A is the predominant β1-adrenoceptor isoform in adipose tissue, skeletal muscle or gut (Evans et al., 1999) whereas a larger proportion of β2D-adrenoceptors are found in the brain (hypothalamus and cortex).

Astrocytes are a major glial cell in the brain. Originally thought to just play a structural role, they have now been shown to have fundamental roles in brain function including (but not limited to) metabolism, transmitter reuptake and release, synaptic transmission and memory formation (Gibbs and Summers, 2002; Gibbs et al., 2006; 2008c; Hutchinson et al., 2007; 2008), with β-adrenoceptor-mediated memory consolidation requiring astrocytic glucogen breakdown and β-adrenoceptor-mediated memory consolidation requiring astrocytic glucose uptake (Hutchinson et al., 2007; Gibbs et al., 2008a,b,c). Although many studies have been conducted using mouse astrocytes there is no information on the presence of β-adrenoceptors, or the function of the three different β-adrenoceptor subtypes in these cells. The use of selective β-adrenoceptor subtype knockout (KO) mice facilitates the identification of the roles of the different subtypes in astrocytic function. This study characterizes the β-adrenoceptor subtypes present in FVB (FVB/N), β1-adrenoceptor knockout (β1KO), DBA/1 crossed with C57BL/6J (DBA × C57) and β2β3-adrenoceptor knockout (β2β3KO) mouse astrocytes and investigates their coupling mechanisms and involvement in glucose uptake.

Methods

Animals

All animal care, breeding and experimental procedures were approved by the Monash University Animal Ethics Committee. Newborn (day 0–1) FVB, β2KO (Susulic et al., 1995), DBA × C57 and β2β3KO mice (Rohrer et al., 1999) were used. The β2KO mice were generated on a FVB background and hence comparisons are made with FVB mice. Because the β2β3KO mice were generated on a 129SVJ, C57Bl6/J and DBA/1 background, comparisons are made with DBA × C57 F1 generation mice. Animals were bred and housed at MouseWorks (Monash University, Clayton) and paired breeding carried out to ensure litters were born at similar times (less than 24 h difference).

Mouse primary astrocyte cell culture

Astrocytes were made from the cerebrum of 0- or 1-day-old mice. Pups were separated from their mothers and used within 30 min. Pups were washed carefully in warmed phosphate buffered saline (PBS) (composition in mM: 136.9 NaCl, 2.7 KCl, 10.1 Na2HPO4, 1.8 KH2PO4, pH 7.4) containing 100 IU·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin and then decapitated. Astrocytes were prepared as previously described (Hutchinson et al., 2007) and cells suspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g·L⁻¹ glucose, 10% fetal bovine serum (FBS), 12.5 mM·L⁻¹...
glutamine, 1 µg·mL⁻¹ amphotericin B, 100 U·mL⁻¹ penicillin and 100 µg·mL⁻¹ streptomycin at 5 mL per brain. Cells were grown in 75 cm² flasks at a density of 10 mL per flask (equivalent to two brains per flask) at 37°C in an atmosphere of 95% air : 5% CO₂ for 1 week with medium changes twice a week. Cells were then shaken at 40 r.p.m. at 37°C overnight in an orbital mixer incubator to remove microglial cells before washing twice with PBS. Astrocytes were loosened from the bottom of flasks using 10 mM EDTA in PBS for 30 min at 37°C and seeded into plates. Astrocytes were grown for another 7 days at 37°C under 5% CO₂, with twice weekly changes of medium.

**Whole cell-binding assay using [³H]-CGP12177A**

Astrocytes were cultured in 48-well plates at a density of 2 x 10⁴ cells per well for 7 days. All experiments were performed at 37°C in a total volume of 100 µL for 1 h as previously described (Hutchinson et al., 2007). For saturation binding experiments, cells were incubated with either [³H]-(+)-4-(3-t-butylamino-2-hydroxypropoxy) benzimidazol-2-one ([³H]-CGP12177A) alone or in the presence of 1 µM (-)-propranolol for the high-affinity binding site or in the presence of 0.1 mM (-)-alprenolol for the low-affinity site to define non-specific binding. [³H]-CGP12177A was used at a concentration of 5–200 pM when examining the high-affinity β₁-β₂-adrenoceptor site and 100–2000 pM for the low-affinity β₁-adrenoceptor site. The hydrophilic radioligand [³H]-CGP12177A was used to measure whole cell binding as it labels cell surface receptors (Staehelin et al., 1983) whereas the hydrophobic [³H]-cyanopindolol labels both cell surface and internalized receptors and is more suited to radioligand binding experiments performed with cell membrane preparations. While both radioligands exhibit little selectivity for β₁- and β₂-adrenoceptors, they have a lower affinity for β₂-adrenoceptors, as do the antagonists alprenolol and propranolol, with high concentrations of alprenolol typically used to define non-specific binding at β₁-adrenoceptors and propranolol typically used to define non-specific binding at β₁- and β₂-adrenoceptors (Durrigan et al., 2000).

Competition experiments were performed at the high-affinity site using a range of concentrations of unlabeled drug (β₁-adrenoceptor antagonist CGP20712A, β₂-adrenoceptor antagonist ICI118551 or β₁-adrenoceptor antagonist SR9230A) and 100 pM of [³H]-CGP12177A with non-specific binding defined by 1 µM (-)-propranolol. Reactions were terminated by aspiration and cells washed twice with PBS. Astrocytes were digested (0.2 M NaOH (200 µL), 50°C), the contents of the wells transferred to scintillation vials and radioactivity counted on a 5% CO₂ for 1 week with medium changes twice a week. The yield and quality of RNA was assessed by measurement of absorbance at 260 and 280 nm and by electrophoresis on 1.0% agarose gel. cDNAs were synthesized by RT of 1 µg of each total RNA using oligo(dT₁₅) as previously described (Roberts et al., 1999).

The PCR amplification cycle number and annealing temperatures for each β-adrenoceptor subtype were: β₁- and β₂-adrenoceptors 32 cycles at 64°C, β₁-adrenoceptor 27 cycles at 60°C, β₂-adrenoceptor splice variants 28 cycles at 64°C; performed on cDNA equivalent of 100 ng starting RNA using primers for β₁-, β₂- or β₁-β₂-adrenoceptors (Invitrogen; Table 1). For all β₁-adrenoceptor PCR, PCR mixes contained 0.5 U Platinum Pfx DNA polymerase (Invitrogen), 1 x Pfx buffer, 1 x enhancer solution, 130 µM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM MgSO₄, 5.8 pmol forward and 5.8 pmol reverse primer. Following amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and images captured digitally.

**[³H]-2-deoxy-D-glucose uptake assay**

Astrocytes were grown in 24-well plates at a density of 4 x 10⁴ cells per well for 7 days. Glucose uptake assays were performed as previously described (Klip et al., 1982; 1984; Tanishita et al., 1997) with some modifications. On day 6, medium was changed to serum free media. The following morning, media was replaced with warmed DMEM (4.5 g·L⁻¹ glucose) and drugs added. After incubation, plates were washed twice with warmed PBS and media changed to warmed glucose-free DMEM. Drugs were re-added for 10 min followed by 10 min incubation with [³H]-2-deoxy-D-glucose (50 nM). While 2-deoxyglucose can inhibit glycolysis and thereby possibly produce anaerobic respiration, this occurs at high concentrations (10–50 mM or higher) and used over a long period of time (hours-days) (Woodward and Hudson, 1954; Jain et al., 1985). Thus, in the current study, we used 50 nM [³H]-2-deoxy-D-glucose over 10 min with uptake being linear over this time (data not shown). Reactions were terminated by aspiration and the cells washed twice with PBS. Samples were digested (0.2 M NaOH, 30 min, 50°C), transferred to scintillation vials and radioactivity counted. Cytochalasin B (10 µM) was used to determine non-facilitated glucose uptake (Birnbaum, 1989).
Table 1
Oligonucleotides used as primers

<table>
<thead>
<tr>
<th>Primer (oligonucleotide)</th>
<th>Expected product size</th>
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<tr>
<td>β-Actin Forward 5′-ATCCCTGCGTATGAGCGGCTTG-3′</td>
<td>441 bp</td>
</tr>
<tr>
<td>β-Adrenoceptor Forward 5′-CGCGCTTACTACAAGCACCAGG-3′</td>
<td>414 bp</td>
</tr>
<tr>
<td>β2-Adrenoceptor Forward 5′-GGTTATCTGGCTGACCACATCTGGTG-3′</td>
<td>468 bp</td>
</tr>
<tr>
<td>ββ-Adrenoceptor Forward 5′-GGTTGCAACTGAGCAGCCTAGTG-3′</td>
<td>384 bp</td>
</tr>
<tr>
<td>ββ-Adrenoceptor (ββ/ββ subtypes) Forward 5′-CCGCACCTTCATAGCATCAAACC-3′</td>
<td>234 bp (ββ)</td>
</tr>
<tr>
<td>Revers 5′-TCTAGTATTCAGCGGAGATTTCTCATC-3′</td>
<td>337 bp (ββ)</td>
</tr>
</tbody>
</table>

Analysis of data
Whole cell binding data was analysed using non-linear curve fitting (GraphPad PRISM version 5.0; GraphPad Software Inc., San Diego, CA, USA) using a one-site model to determine Kd and Bmax (saturation binding experiments) or non-linear curve fitting for one-site competition experiments to obtain pKb values using the Cheng and Prussoff equation (Cheng and Prussoff, 1973). A standard curve from cAMP standards (10⁻⁶ M to 10⁻¹¹ M) was generated using GraphPad PRISM and points read from standard curve to determine cAMP accumulation. Data was normalized to the forskolin (10⁻⁶ M) response in each respective plate. Statistical significance was determined using two-way analysis of variance (ANOVA) or Student's t-test. P-values < 0.05 were considered significant. Where appropriate, pKb values were calculated using the method of Furchgott (1972) where pKb = log(dose ratio – 1) – log[agonist]. Glucose uptake experiments were performed in triplicate and data were analysed using non-linear least square fitting (GraphPad PRISM version 5.0) to obtain pEC50 values. All results are expressed as mean ± SEM of n experiments.

Materials
(±)-CGP20712A (Dr G Anderson, Ciba-Geigy AG, Basel) and zinterol hydroxido (Bristol-Myers Squibb, Noble Park, Vic, Australia) were gifts. Other materials were purchased as follows: [³H] 2-deoxyglucose (specific activity 8.0 Ci·nmol⁻¹) (PerkinElmer Life and Analytical Sciences, Boston, MA, USA); [³H]-CGP12177A (specific activity 50 Ci·nmol⁻¹) (GE Healthcare, Little Chalfont, UK); (±)-IC118551 (Imperial Chemical Industries, Wilmslow, Cheshire, UK); 10 × RT buffer, dNTPs, oligo(dT)₁₅, RNasin, reverse transcriptase, 10 × PCR buffer, Taq polymerase, enhancing solution, oligonucleotides (Invitrogen, Carlsbad, CA, USA); insulin (Actrapid®) (NOVO Nordisk Pharmaceuticals Pty Ltd, Baulkham Hills, NSW, Australia); (−)-alprenolol, CL316243, cytochalasin B, deoxyribonuclease I from bovine pancreas type IV, forskolin, IBMX (−)-isoprenaline, PTX, polyethyleneimine (−)-propranolol, SR59230A, trypsin from porcine pancreas Type IX-S, trypsin inhibitor from soybean type II-S (Sigma Chemical Company, St Louis, MO, USA); amphotericin B, Hank's balanced salt solution (Invitrogen Corporation, Carlsbad, CA, USA); FBS (JRH Biosciences Inc, Lexana, KS, USA); all other cell culture reagents obtained from Trace Biosciences (Castle Hill, NSW, Australia). All other drugs and reagents were of analytical grade.

Results
Expression of β₁-, β₂- and β₃-adrenoceptor mRNA in mouse astrocytes
RT-PCR was carried out to determine the β-adrenoceptor subtypes that are expressed in mouse astrocytes with brown fat and brain from an FVB mouse used as positive controls, as both these tissues express all three β-adrenoceptor subtypes. β₁- and β₃-adrenoceptor mRNA were detected in FVB, DBA × C57 and β₁KO astrocytes and β₃-adrenoceptor mRNA was present in FVB, DBA × C57 and β₁β₃KO astrocytes (Figure 1A). This also confirmed that the β₁β₃-KO and β₁-KO mice were of the correct genotype. The mouse β₁-adrenoceptor gene contains two exons that undergo alternative splicing to produce two splice variants of the mouse β₁-adrenoceptor that are expressed, the β₁-adrenoceptor and the β₁β₁-adrenoceptor (Evans et al., 1999). Using intron-spanning primers that detect both transcripts, it appears that the β₁β₁-adrenoceptor is the predominant isoform in mouse astrocytes (Figure 1B).

[³H]-CGP12177A binding in mouse astrocytes
Receptor binding was used to determine the level of β-adrenoceptor protein expression. A high-affinity β₁β₁-adrenoceptor site was demonstrated in astrocytes from all mouse strains (FVB pKd 9.57 ± 0.28, Bmax 96.35 ± 30.59 fmol·mg⁻¹ protein, n = 14; β₁KO pKd 9.79 ± 0.27, Bmax 89.61 ± 23.09 fmol·mg⁻¹ protein, n = 12; DBA × C57 pKd 9.61 ± 0.17, Bmax 95.8 ± 41.3 fmol·mg⁻¹ protein, n = 5) except β₁β₃-KO astrocytes (Figure 2), confirming that the high-affinity
β1-Adrenoceptor saturation experiments demonstrated a low-affinity binding site in astrocytes from all mouse strains including β2KO mice (FVB pKᵋ = 9.12 ± 0.05, Bₘₐₓ 224.5 ± 105.9 fmol·mg⁻¹ protein, n = 5; β1KO pKᵋ = 9.15 ± 0.14, Bₘₐₓ 303.5 ± 118.7 fmol·mg⁻¹ protein, n = 5; DBA × C57 pKᵋ = 9.15 ± 0.02, Bₘₐₓ 128.8 ± 66.8 fmol·mg⁻¹ protein, n = 3; β1β2KO pKᵋ = 9.19 ± 0.32, Bₘₐₓ 54.5 ± 13.7 fmol·mg⁻¹ protein, n = 3) (Figure 4). The low-affinity binding site in astrocytes from β1KO mice cannot be due to β2-adrenoceptors since no mRNA was present (Figure 1), but could be due to [³H]-CGP12177A binding to a low-affinity state of the β1-adrenoceptor (Konkar et al., 2000).

Effect of the general β-adrenoceptor agonist isoprenaline, the β2-adrenoceptor agonist zinterol and the selective β₁-adrenoceptor agonist CL316243 on cAMP accumulation in mouse astrocytes

The general β-adrenoceptor agonist isoprenaline increased cAMP accumulation in astrocytes from all mouse strains except β1β2KO mice (pEC₅₀ FVB 8.44 ± 0.4, n = 18; β1KO 8.64 ± 0.4, n = 15; DBA × C57 8.35 ± 0.3, n = 16; β1β2KO not determined, n = 10), suggesting that isoprenaline increases cAMP accumulation by activating β1- or β2-adrenoceptors. Zinterol, a β2-adrenoceptor agonist, increased cAMP accumulation in FVB, β1KO and DBA × C57 mouse astrocytes, but not in β1β2KO astrocytes (pEC₅₀ FVB 7.02 ± 0.4, n = 12; β1KO 7.88 ± 0.5, n = 14; DBA × C57 6.98 ± 0.4, n = 10; β1β2KO not determined, n = 10) suggesting that zinterol increases cAMP accumulation by activating β2-adrenoceptors. The β1-adrenoceptor agonist CL316243 failed to increase cAMP accumulation in astrocytes from any mouse strain at any concentration used (n = 13–17) (Figure 5).

Effect of selective β-adrenoceptor antagonists on cAMP accumulation after stimulation by the general β-adrenoceptor agonist isoprenaline in mouse astrocytes

Concentration response curves to isoprenaline were performed in the absence and presence of selective β-adrenoceptor antagonists (β1-adrenoceptor, CGP20712A; β2-adrenoceptor, ICI118551; β1-adrenoceptor, SR59230A) at a concentration of 300 nM. None of these antagonists alone affected cAMP accumulation (data not shown). CGP20712A antagonized the response to isoprenaline more effectively than ICI118551 or SR59230A in astrocytes from all strains of mice except for β1β2KO mice that failed to show significant cAMP accumulation (Table 3; Figure 6). This suggests that β2-adrenoceptors are the predominant adrenoceptor responsible for the isoprenaline-mediated increases in cAMP accumulation.

Effect of CL316243 on cAMP accumulation in mouse astrocytes in the absence and presence of forskolin

To investigate if β2-adrenoceptors were weakly coupled to Gₐ, forskolin was used to prime cells before the addition of CL316243 (Figure 7). In FVB astrocytes, CL316243 alone had no significant effect on cAMP accumulation. However when
primed with forskolin, cAMP accumulation increased in a concentration-dependent manner [control pEC50 not determined; +forskolin pEC50 7.83 ± 0.4, max response (maximum cAMP accumulation achieved expressed as a percentage of the response to forskolin) 175 ± 15, n = 10]. In β3KO astrocytes CL36243 failed to increase cAMP accumulation in the absence or presence of forskolin (n = 10), which suggested that the effect observed in FVB astrocytes was due to β3-adrenoceptors. The DBA × C57 astrocytes showed a similar response to that seen in FVB astrocytes (data not shown). This suggests weak coupling of the β3-adrenoceptor to Gi.

**Effect of the G<sub>i</sub> inhibitor PTX on cAMP accumulation in response to isoprenaline in mouse astrocytes**

Mammalian β-adrenoceptors can couple to both G<sub>i</sub> and G<sub>q</sub> (Soeder et al., 1999; Hutchinson et al., 2002; 2007; Sato et al., 2005); therefore, the role of G<sub>i</sub> was investigated in mouse astrocytes using PTX (100 ng·mL<sup>-1</sup> overnight) to inhibit G<sub>i</sub>. FVB astrocytes treated with PTX demonstrated an enhanced response to isoprenaline compared with isoprenaline alone (control pEC50 8.40 ± 0.9, max response 25.7 ± 3.8; +PTX pEC50 8.43 ± 0.6, max response 41.0 ± 4.4; n = 7, *P < 0.05 two-way ANOVA) (Figure 8). The β1β2KO astrocytes showed a similar response to PTX and isoprenaline treatment to FVB astrocytes (data not shown). There was still no observable effect of isoprenaline alone on cAMP accumulation although, after treatment with PTX, high concentrations of isoprenaline appeared to cause cAMP accumulation in β1β2KO astrocytes (data not shown).

**Figure 2**

Saturation binding of [3H]-CGP12177A to a high-affinity site in whole astrocytes isolated from (A) FVB (FVB/N) (n = 14), (B) β3-adrenoceptor knockout (β3KO) (n = 12), (C) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 5) and (D) β1β2-adrenoceptor knockout (β1β2KO) (n = 3) mice. Incubations were performed for 60 min at 37°C and non-specific binding defined by (–)-propranolol (1 μM). Points show mean ± SEM of experiments performed in duplicate. Results are expressed as specific binding in fmol·mg<sup>-1</sup> protein. A high-affinity binding site was present in astrocytes from all mouse strains except for the β1β2KO mice indicating that the majority of receptors are β1- or β2-adrenoceptors at this concentration of [3H]-CGP12177A.
Effect of CL316243 on cAMP accumulation in mouse astrocytes in the absence and presence of forskolin and/or PTX

The coupling of β3-adrenoceptors to Gi was further investigated by constructing concentration-response curves to CL316243 in the absence and presence of forskolin and/or PTX. As previously observed, CL316243 alone did not increase cAMP accumulation either in the presence or absence of PTX. When cells were primed with a submaximal concentration of forskolin, CL316243 was able to increase cAMP accumulation in a concentration-dependent manner, and this effect was enhanced with prior PTX treatment (Figure 9) (+forskolin pEC50 7.17 ± 0.4, max response 224.2 ± 31.5; +forskolin & PTX pEC50 7.56 ± 0.3, max response 282.2 ± 24.2; n = 4, *P < 0.05 two-way ANOVA between CL316243 + forskolin and CL316243 + forskolin and PTX).

Effect of β-adrenoceptor stimulation on glucose uptake in mouse primary astrocytes

Insulin increased glucose uptake in a concentration-dependent manner in all four strains of mouse astrocytes with no significant differences between wild type and respective KO (pEC50 FVB 9.01 ± 0.23, n = 7; β3KO 8.79 ± 0.32, n = 7; DBA × C57 8.99 ± 0.29, n = 8; β1β2KO 9.22 ± 0.31, n = 9) (Figure 10). Isoprenaline increased glucose uptake in a concentration-dependent manner in all four strains of mouse astrocytes (pEC50 FVB 8.88 ± 0.3, n = 18; β3KO 8.37 ± 0.4, n = 13; DBA × C57 8.89 ± 0.3, n = 16; β1β2KO 8.71 ± 0.3, n = 14) (Figure 11). The β3-adrenoceptor agonist zinterol increased glucose uptake in FVB, β3KO and DBA × C57 astrocytes but this effect was not present in β1β2KO astrocytes (pEC50 FVB 9.58 ± 0.2, n = 18; β3KO 8.99 ± 0.3, n = 13; DBA × C57 9.15 ± 0.3, n = 11; β1β2KO not determined, n = 10). The β3-adrenoceptor agonist

### Table 3

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Antagonist (300 nM)</th>
<th>pKb</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB</td>
<td>CGP20712A (β1-adrenoceptor)</td>
<td>8.61 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>ICI118551 (β2-adrenoceptor)</td>
<td>7.40 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>SR59230A (β3-adrenoceptor)</td>
<td>7.40 ± 0.20</td>
</tr>
<tr>
<td>β3KO</td>
<td>CGP20712A</td>
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<td>ICI118551</td>
<td>6.82 ± 0.30</td>
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<td></td>
<td>SR59230A</td>
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<tr>
<td>DBA × C57</td>
<td>CGP20712A</td>
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<td></td>
<td>ICI118551</td>
<td>6.92 ± 0.10</td>
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<tr>
<td></td>
<td>SR59230A</td>
<td>7.54 ± 0.20</td>
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</tbody>
</table>

Values are mean pKb values ± SEM of seven experiments performed.

DBA × C57, DBA/1 crossed with C57BL/6J; FVB, FVB/N; β3KO, β3-adrenoceptor knockout.
CL316243 increased glucose uptake in all mouse astrocyte strains except β3KO (pEC50 FVB 9.95 ± 0.4, n = 20; β3KO not determined, n = 8; DBA × C57 8.85 ± 0.4, n = 6; β1β2KO 8.65 ± 0.4, n = 9). There was no significant difference in glucose uptake after treatment with isoprenaline or zinterol between FVB and β3KO astrocytes (two-way ANOVA P = 0.89, P = 0.71, respectively). Similar results were observed for DBA × C57 and β1β2KO astrocytes after treatment with isoprenaline and CL316243 (two-way ANOVA P = 0.31, P = 0.50, respectively). This suggests that β2- and β3-adrenoceptors increase glucose uptake in mouse astrocytes although the role of β1-adrenoceptors in this process is yet to be defined.

Discussion

β-Adrenoceptors have been identified on mammalian astrocytes in several studies (Voisin et al., 1987; Salm and McCarthy, 1989; Shao and Sutin, 1992; Junker et al., 2002; Mori et al., 2002; Tanaka et al., 2002; Ghosh and Das, 2007) although the receptor subtype present is generally not well defined apart from responses typically being mediated by the non-selective β-adrenoceptor agonist isoprenaline and blocked by the non-selective β-adrenoceptor antagonist propranolol. Hence in this study we have aimed to identify which β-adrenoceptor subtypes are present in astrocytes using β-adrenoceptor selective KO mice and to verify whether β-adrenoceptor stimulation increases glucose uptake in mammalian astrocytes.

Previous studies demonstrated mRNA expression of all three β-adrenoceptor subtypes in chick astrocytes together with β1- and β2-adrenoceptor protein (Hutchinson et al., 2007). In the present study, β1- and β3-adrenoceptor mRNA was identified in astrocytes from all mouse strains tested, except β1β3KO; and β1-adrenoceptor mRNA (including β1- and β2-adrenoceptor mRNA) in all strains except for β3KO. While β1- and β2-adrenoceptor mRNA have been demonstrated in rat cultured astrocytes (Joardar et al., 2006; Ghosh and Das, 2007) β3-adrenoceptor mRNA has not, even

Figure 4
Saturation binding of [3H]-CGP12177A to low-affinity site in whole astrocytes isolated from (A) FVB (FVB/N) (n = 5), (B) β3-adrenoceptor knockout (β3KO) (n = 5), (C) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 4) and (D) β1β2-adrenoceptor knockout (β1β2KO) (n = 3) mice. Incubations were performed for 60 minutes at 37°C and non-specific binding defined by (-)-alprenolol (0.1 mM). Points show mean ± SEM of experiments performed in duplicate. Results expressed as specific binding in fmol·mg⁻¹ protein. A low-affinity site is present in primary astrocytes from all mouse strains.
though it is present in rat (Summers et al., 1995) and mouse brain regions (Evans et al., 1999). The mouse β3-adrenoceptor occurs as two splice variants, β3A-adrenoceptor and β3B-adrenoceptor (Evans et al., 1999), that share identical pharmacological but different signalling properties (Hutchinson et al., 2002; Sato et al., 2005; 2007; 2008). The β3B-adrenoceptor has higher expression in the brain compared to other tissues (Evans et al., 1999) but in astrocytes, the present study identified both β3A- and β3B-adrenoceptor, with β3A-adrenoceptor predominating.

Radioligand binding studies displayed high-affinity sites in astrocytes from FVB, β3KO and DBA × C57 mice, but not β1β2KO mice. Bmax values (~90 fmol·mg−1 protein) were comparable with studies in rat cultured astrocytes; 20–30 fmol·mg−1 protein from rat cerebra (Joardar et al., 2006); ~30 fmol·mg−1 protein from rat forebrain (Sapena et al., 1996); 10–60 fmol·mg−1 protein in rat astrocytes (Ghosh and Das, 2007); 140–210 fmol·mg−1 protein from rat cerebral astrocytes (Das and Paul, 1994). Competition binding studies showed CGP20712A to have the highest affinity confirming that β3-adrenoceptors predominate (Sapena et al., 1996) whereas β1-adrenoceptors predominate in chick astrocytes (Hutchinson et al., 2007). The β2-adrenoceptor antagonist ICI118551 also competed for this site, albeit with an affinity intermediate between that expected for β2- and β1-adrenoceptors, suggesting that a small population of β2-adrenoceptors exist in mouse astrocytes. Saturation binding studies also showed a low-affinity site in all mouse strains including β3KO astrocytes. This is likely to reflect binding of [3H]-CGP12177A to the low-affinity form of the β3-adrenoceptor (Pak and Fishman, 1996). This compares well to our study where [3H]-CGP12177A only binds to the low-affinity site in astrocytes derived from β3β3KO mice. β3-adrenoceptors cannot be responsible for the low-affinity site shown in β3KO astrocytes as β3-adrenoceptor mRNA is not present in these cells, however a low-affinity β1-adrenoceptor site may account for our results.

Figure 5
The effect of the β3-adrenoceptor agonist isoprenaline, the β2-adrenoceptor agonist zinterol and the selective β3-adrenoceptor agonist CL316243 on cAMP accumulation in (A) FVB (FVB/N), (B) β3-adrenoceptor knockout (β3KO), (C) DBA/1 crossed with C57BL/6J (DBA × C57) and (D) β1β2-adrenoceptor knockout (β1β2KO) astrocytes. Results expressed as a % of response to forskolin (10−4 M). Each point shows mean ± SEM (n = 10–18).
All three β-adrenoceptor subtypes couple to Gs to activate adenylate cyclase and increase intracellular cAMP levels. The non-selective β-adrenoceptor agonist isoprenaline, increased cAMP accumulation in FVB, β3KO and DBA ¥ C57 astrocytes but not β1β2KO astrocytes, indicating that isoprenaline increases cAMP through β1- or β2-adrenoceptors. Isoprenaline concentration-response curves were strongly antagonized by CGP20712A with pKB values (8.3–8.6; Table 3) appropriate for antagonism at β1-adrenoceptors and not β2- or β3-adrenoceptors. Isoprenaline concentration-response curves were strongly antagonized by CGP20712A with pKα values (8.3–8.6; Table 3) appropriate for antagonism at β1-adrenoceptors and not β2- or β3-adrenoceptors where pKB values are significantly lower (Table 4). The β2-adrenoceptor antagonist ICI118551 weakly antagonized the isoprenaline response (pKα 6.8–7.4, Table 3), with a pKα value lower than its known value for β2-adrenoceptor mediated responses (Table 4). SR59230A, first described as a β3-adrenoceptor antagonist (Nisoli et al., 1996), antagonized isoprenaline responses with pKα values of 7.4–7.5 (Table 3), lower than expected for β3-adrenoceptors that may reflect β3-adrenoceptor antagonist actions (Table 4) (Manara et al., 1996; Hutchinson et al., 2001). However, in other studies SR59230A (<1 μM) did not affect salbutamol (β2-adrenoceptor) or dobutamine (β1-adrenoceptor) mediated cAMP accumulation in rat cerebellum or frontal cortex (Nisoli et al., 1996) and failed to antagonize noradrenaline-mediated cAMP generation in brown preadipocytes (predominately β3-adrenoceptor), but did abolish noradrenaline-mediated effects in mature brown adipocytes (predominately β3-adrenoceptor) (Nisoli et al., 1996). Because pKα values for SR59230A were similar in FVB and β3-adrenoceptor KO astrocytes it is likely that SR59230A antagonizes isoprenaline-mediated cAMP accumulation in these cells by blocking β3-adrenoceptors. Thus, β1-adrenoceptors play a greater role than β2- or β3-adrenoceptors in isoprenaline-mediated cAMP accumulation in mouse astrocytes. Antagonist pKα values did not differ between FVB and β3KO astrocytes (pEC50 values

**Figure 6**

Effect of the selective β1-adrenoceptor antagonist CGP20712A, the selective β2-adrenoceptor antagonist ICI118551 and the selective β3-adrenoceptor antagonist SR59230A in (A) FVB (FVB/N) (n = 7), (B) β3-adrenoceptor knockout (β3KO) (n = 7), (C) DBA/1 crossed with C57BL/6J (DBA ¥ C57) (n = 7) and (D) β1β2-adrenoceptor knockout (β1β2KO) (n = 9) mouse primary astrocytes on cAMP accumulation in response to isoprenaline. Concentrations of antagonist used were 300 nM. Results expressed as a percentage of forskolin (10⁻⁴ M). Each point shows mean ± SEM.
### Table 4

Affinity values for β-adrenoceptor antagonists at the β₁-, β₂- or β₃-adrenoceptor

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>β₁-Adrenoceptor</th>
<th>β₂-Adrenoceptor</th>
<th>β₃-Adrenoceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGP20712A</td>
<td>9.0* (Molenaar and Summers, 1987)</td>
<td>5.9 (Molenaar and Summers, 1987)</td>
<td>4.8 (Hollenga and Zaagsma, 1989)</td>
</tr>
<tr>
<td>ICI118551</td>
<td>7.2 (Bilski et al., 1983)</td>
<td>8.4–9.6 (Nevzorova et al., 2002)</td>
<td>5.9–6.0 (Nisoli et al., 1996)</td>
</tr>
<tr>
<td>SRS9230A</td>
<td>7.4 (Hutchinson et al., 2001)</td>
<td>6.6 (Manara et al., 1996)</td>
<td>8.2–8.9 (Nisoli et al., 1996)</td>
</tr>
</tbody>
</table>

*pA₂ values, all other values are pKᵦ values.

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**Figure 7**

Effect of the selective β₁-adrenoceptor agonist CL316243 and CL316243 in the presence of forskolin (10 μM) on cAMP accumulation in astrocytes from (A) FVB (FVB/N) (n = 10) and (B) β₁-adrenoceptor knockout (β₁KO) (n = 10). Results expressed as a percentage of forskolin (10⁻⁴ M). Each point shows mean ± SEM. Note that in astrocytes containing β₁-adrenoceptors, the response to CL316243 is enhanced in the presence of forskolin.

**Figure 8**

Effect of isoprenaline and isoprenaline + Pertussis toxin (PTX; 100 ng·mL⁻¹ overnight) on cAMP accumulation in (A) FVB (FVB/N) (n = 7) and (B) β₁-adrenoceptor knockout (β₁KO) (n = 4) mouse astrocytes. Results expressed as a percentage of forskolin (10⁻⁴ M). Each point shows mean ± SEM.
for isoprenaline were also similar) suggesting that β₁-adrenoceptors do not compensate for β₂-adrenoceptors as in ileum from β₁-adrenoceptor KO mice where β₁-adrenoceptor mRNA and protein levels are elevated (Hutchinson et al., 2001). Conversely, in brown adipocytes from β₁-adrenoceptor KO mice, β₁- and α₁-adrenoceptors functionally compensate for β₁-adrenoceptors without altering mRNA or protein levels (Chernogubova et al., 2005). We however saw no compensation in β₁KO astrocytes by β₂- or β₁-adrenoceptors based on mRNA, protein or functional results.

The β₁-adrenoceptor agonist zinterol increased cAMP levels in a concentration-dependent manner in FVB, β₁KO and DBA × C57 astrocytes but not β₁β₂KO astrocytes, indicating that zinterol increases cAMP through β₁- or β₂-adrenoceptors. This is likely to be due to β₂-adrenoceptor stimulation since responses were abolished in β₁β₂KO astrocytes and only one previous study has shown zinterol to act at β₂-adrenoceptors and increase glucose uptake and cAMP levels in brown adipocytes derived from β₁-adrenoceptor KO mice, where β₁-adrenoceptors functionally compensate for lack of β₂-adrenoceptors (Hutchinson et al., 2006). Zinterol can also act as a β₁-adrenoceptor agonist in some circumstances (Hutchinson et al., 2006) although this is not likely here since responses were identical in FVB versus β₁KO astrocytes. This suggests, along with the radioligand binding results, a small population of functional β₁-adrenoceptors in mouse astrocytes.

The β₁-adrenoceptor agonist CL316243 alone was unable to stimulate cAMP accumulation in any of the mouse astrocytes strains used, indicating that β₁-adrenoceptors may not couple strongly enough to Gs, in this system to elicit a CAMP response. β₁-Adrenoceptors can also couple to Gs in several systems (Chaudhry et al., 1994; Soeder et al., 1999; Hutchinson et al., 2002; Sato et al., 2005; 2008). Our results show that β₁-adrenoceptors in mouse astrocytes can weakly couple to Gs, and Gs, based on two different types of experiments. To investigate if β₁-adrenoceptors were Gs, coupled, astrocytes were pretreated with the Gs inhibitor PTX before stimulation with isoprenaline. The FVB astrocytes treated with PTX demonstrated an enhanced CAMP response to isoprenaline whereas PTX had no effect on the isoprenaline mediated CAMP response in β₁KO astrocytes indicating that β₁-adrenoceptors are coupled to Gs. To investigate if β₁-adrenoceptors can couple weakly to Gs, astrocytes were treated with CL316243 in the absence and presence of forskolin, a direct adenylate cyclase stimulator. In FVB mouse astrocytes, CL316243 had no effect on its own but when primed with forskolin, cAMP accumulation increased in a concentration dependent manner (pEC50 8.5 ± 0.1). This pEC50 value compares well to other studies in cells and tissues expressing moderate-high levels of β₁-adrenoceptors (Hutchinson et al., 2001; 2006; Chernogubova et al., 2005) and suggests that β₁-adrenoceptors are weakly coupled to Gs. CL316243 had no effect on CAMP accumulation in β₁KO mouse astrocytes in the absence or presence of forskolin, confirming this effect is dependent upon the presence of the β₁-adrenoceptor. Assays for CAMP revealed an increased response to CL316243 in the presence of forskolin in β₁β₂KO astrocytes as compared to

**Figure 9**

Effect of the selective β₁-adrenoceptor agonist CL316243, CL316243 in the presence of forskolin (10 µM), CL316243 in the presence of Pertussis toxin (PTX) and CL316243 in the presence of PTX and forskolin on CAMP accumulation in astrocytes from FVB (FVB/N) mice. Results expressed as a percentage of forskolin (10^{-4} M). Each point shows mean ± SEM of 4 experiments.

**Figure 10**

Effect of insulin on [³H] 2-deoxy-D-glucose uptake in whole astrocytes from (A) FVB (FVB/N) (n = 21), β₁-adrenoceptor knockout (β₁KO) (n = 7), (B) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 8) and β₁β₂-adrenoceptor knockout (β₁β₂KO) (n = 9) mice. Results expressed as a percentage of basal and normalized to 100% in each experiment. Each point shows mean ± SEM.
Figure 11
The effect of (A, B) isoprenaline (C, D) zinterol or (E, F) CL316243 on [3H]2-deoxy-D-glucose uptake in whole astrocytes from FVB (FVB/N) (n = 18–20) and β3-adrenoceptor knockout (β3KO) (n = 8–13) (A, C, E), and DBA/1 crossed with C57BL/6J (DBA × C57) (n = 6–16) and β1β2-adrenoceptor knockout (β1β2KO) (n = 9–14) (B, D, F) mice. Results are expressed as a percentage of the basal response normalized to 100% in each experiment. Each point shows mean ± SEM. Note there was no glucose uptake after treatment with zinterol in the β1β2KO astrocytes and no glucose uptake after treatment with CL316243 in β3KO astrocytes.
DBA × C57 astrocytes which may indicate a ‘supranormal’ response to β₂-adrenoceptor agonists as observed by (Rohrer et al., 1999). In β₁β₂KO mice there is a supranormal response to CL316243 in cardiac tissues, which may be due to upregulation of vascular β₁-adrenoceptors in these mice (Rohrer et al., 1999).

Glucose uptake is stimulated by β₂-adrenoceptors in skeletal muscle (Nevzorova et al., 2002) and by β₁-adrenoceptors in brown adipocytes (Chernogubova et al., 2005). β₁-adrenoceptors increase glucose uptake in chick astrocytes (Hutchinson et al., 2007; Gibbs et al., 2008c), and in rat astrocyte-enriched cultures (Hsu and Hsu, 1990). Here we demonstrated that isoprenaline, zinterol and CL316243 all increased glucose uptake in wildtype mouse astrocytes. The zinterol effect was abolished in β₁β₂KO astrocytes indicating that it probably acted via β₂-adrenoceptors. The effect cannot be due to actions at β₁-adrenoceptors (Hutchinson et al., 2006) because zinterol responses were intact in β₁KO astrocytes. Additionally CL316243 increased glucose uptake in all astrocyte strains except β₁-KO astrocytes, indicating that β₁-adrenoceptors can also increase glucose uptake. Glucose uptake can follow activation of different intracellular signalling mechanisms on β₁-adrenoceptor stimulation (Chernogubova et al., 2005; Nevzorova et al., 2006; Hutchinson et al., 2007) which may or not involve coupling to G. In chick astrocytes, both β₁- and β₂-adrenoceptors increase glucose uptake (Hutchinson et al., 2007; 2008) with β₂-adrenoceptors increasing glucose uptake over longer periods of time (due to glycogen depletion) through a Gi-mediated mechanism, whereas β₁-adrenoceptors increase glucose uptake rapidly through a Gs-mediated mechanism (Hutchinson et al., 2007). However, the mechanism involved in β₁-adrenoceptor stimulation of glucose uptake in mouse astrocytes has not been examined here in this study and needs further investigation.

A limitation of the present study is the use of whole cerebrum because astrocytes from different brain regions can be morphologically distinct (Pinto et al., 2000), and express varying levels of transporters, receptors and signal transduction molecules (Hansson, 1988), including the 1- and 2-adrenoceptors (Ernsberger et al., 1990). Activation of β₁-adrenoceptors may therefore involve different signalling pathways depending upon signalling molecules expressed in astrocytes from different brain regions, thus exhibiting region-specific coupling as described for other receptors (Bianco et al., 2009).

This study identified all β₁-adrenoceptor mRNA subtypes, including β₁-adrenoceptor splice variants, in wildtype mouse astrocytes. β₁-adrenoceptors are the predominant subtype responsible for the cAMP response with a minor β₂-adrenoceptor component. β₂-Adrenoceptors are coupled to G and weakly to G, whereas β₁- and β₂-adrenoceptors only couple to G. This study demonstrates that all β₁-adrenoceptors are involved in glucose uptake although further investigation is required into the signalling mechanisms involved.

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Conflict of interest

None.

References


β-Adrenoceptors in mouse astrocytes


