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RESEARCH PAPER

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

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BACKGROUND AND PURPOSE

β_1-, β_2- and β_3-adrenoceptors determined by functional, binding and reverse transcription polymerase chain reaction (RT-PCR) studies are present in chick astrocytes and activation of β_2- or β_3-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, β_3-adrenoceptor knockout and $\beta_1\beta_2$-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 β_1- and β_3-adrenoceptors were predominant. cAMP studies showed that β_1- and β_2-adrenoceptors coupled to G_s whereas β_3-adrenoceptors coupled to both G_s and G_i. 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 β_1-adrenoceptors are the predominant β-adrenoceptor in mouse astrocytes and are primarily responsible for cAMP production in response to β-adrenoceptor stimulation, β_3-adrenoceptors are also present in mouse astrocytes and activation of β_2- and β_3-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)-1*H*-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethanesulphonate; CL316243 (R,R)-5-[2-[[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-[(1**S**)-1,2,3,4-tetrahydronapth-1-ylamino]-2**S**-2-propanol oxalate

Introduction

Adrenoceptors are G protein-coupled receptors (GPCRs) comprising three types: α_1-, α_2- and β-adrenoceptors (nomenclature follows Alexander *et al.*, 2009). α_1-Adrenoceptors couple to G_q to activate phospholipase C and increase inositol trisphosphate and diacylglycerol; α_2-adrenoceptors couple to G_i to negatively regulate adenylyl cyclase and cAMP levels; whereas β-adrenoceptors couple to G_s to positively regulate adenylyl cyclase to increase intracellular cAMP levels (Alexander *et al.*, 2009). While β-adrenoceptors primarily couple to G_s, coupling to G_i has also been documented for the β_2- (Daaka *et al.*, 1997; Xiao, 2001; Woo *et al.*, 2009) and β_3-adrenoceptors (Hutchinson *et al.*, 2002; 2007; Sato *et al.*, 2005; 2007). β_{3a}- and β_{3b}-adrenoceptors are splice variants of the β_3-adrenoceptor (Evans *et al.*, 1999) that show identical pharmacological profiles (Hutchinson *et al.*, 2002), but the β_{3a}-adrenoceptor couples solely to G_s whereas the β_{3b}-adrenoceptor couples both to G_s and G_i (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, β_1- and β_2-adrenoceptors are expressed at similar densities but β_1-adrenoceptors predominate in the cerebellar cortex, hippocampus and basal ganglia (Paschalis *et al.*, 2009) whereas β_2-adrenoceptors predominate in the cerebellum and thalamus (Rainbow *et al.*, 1984). β_3-Adrenoceptors in the rodent brain are limited to the hippocampus, hypothalamus, amygdala and cerebral cortex (Summers *et al.*, 1995). β_3-Adrenoceptors have roles in the regulation of food intake (Tsujii and Bray, 1998) and for the treatment of anxiety and depressive disorders (Stemmelin *et al.*, 2008) and activation following intraventricular injection of β_3-adrenoceptor agonists increases c-fos expression in the hypothalamus (Castillo-Melendez *et al.*, 2000). The β_{3a}- is the predominant β_3-adrenoceptor isoform in adipose tissue, skeletal muscle or gut (Evans *et al.*, 1999) whereas a larger proportion of β_{3b}-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 *et al.*, 2008c). Early studies of rodent astrocytes and glial cells identified β-adrenoceptors (Salm and McCarthy, 1989; Shao and Sutin, 1992) but subtype distribution is unclear, with some studies in glial cells indicating either β_2-adrenoceptors (Shao and Sutin, 1992), β_1-adrenoceptors (Sapena *et al.*, 1996) or a mixed population of both β_1- and β_2-adrenoceptors (Voisin *et al.*, 1987; Junker *et al.*, 2002; Mori *et al.*, 2002; Tanaka *et al.*, 2002; Ghosh and Das, 2007). β-Adrenoceptors have a variety of roles in astrocytes including regulation of cellular morphology (Shain *et al.*, 1987), protection against glutamate-induced cell death (Junker *et al.*, 2002) and protection against inflammatory mediators (Abdulla and Renton, 2005). Studies investigating β-adrenoceptor mediated regulation of glucose and glycogen metabolism on mammals are sparse but include β-adrenoceptor-regulation of glycogenolysis (Sorg and Magistretti, 1991; O'Dowd *et al.*, 1995) and β-adrenoceptor-mediated increases in glucose uptake in glial cells (Hsu and Hsu, 1990). The possibility that β_3-adrenoceptors occur in glial cells has received very little attention; β_3-adrenoceptor mRNA was absent in rat cultured microglia (Tanaka *et al.*, 2002) but both β_3-adrenoceptor mRNA and protein were present in chick astrocytes (Hutchinson *et al.*, 2007).

In chick astrocytes, a role for β-adrenoceptors in glucose uptake has been established with both β_2-adrenoceptors and β_3-adrenoceptors regulating this process through a G_s- and G_i-dependent pathway, respectively (Hutchinson *et al.*, 2007). Glucose metabolism is important for many functions in the brain, including cognition, memory and learning (Messier, 2004). We have established that glucose and glycogen metabolism are important for memory formation in the day-old chicks (Gibbs and Summers, 2002; Gibbs *et al.*, 2006; 2008c; Hutchinson *et al.*, 2007; 2008), with β_2-adrenoceptor-mediated memory consolidation requiring astrocytic glycogen breakdown and β_3-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_3-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), β_3-adrenoceptor knockout (β_3KO), DBA/1 crossed with C57BL/6J (DBA × C57) and $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) 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, β_3KO (Susulic *et al.*, 1995), DBA × C57 and $\beta_1\beta_2$KO mice (Rohrer *et al.*, 1999) were used. The β_3KO mice were generated on a FVB background and hence comparisons are made with FVB mice. Because the $\beta_1\beta_2$KO 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 Na_2HPO_4, 1.8 KH_2PO_4, 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-

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 cm^2 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% CO_2 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% CO_2, with twice weekly changes of medium.

Whole cell-binding assay using [^{3}H]-CGP12177A

Astrocytes were cultured in 48-well plates at a density of 2 × 10^4 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 [^{3}H]-(±)-4-(3-t-butylamino-2-hydroxypropoxy) benzimidazol-2-one ([^{3}H]-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. [^{3}H]-CGP12177A was used at a concentration of 5–200 pM when examining the high-affinity $\beta_1\beta_2$-adrenoceptor site and 100–2000 pM for the low-affinity β_3-adrenoceptor site. The hydrophilic radioligand [^{3}H]-CGP12177A was used to measure whole cell binding as it labels cell surface receptors (Staehelin *et al.*, 1983) whereas the hydrophobic [^{125}I]-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 β_1- and β_2-adrenoceptors, they have a lower affinity for β_3-adrenoceptors, as do the antagonists alprenolol and propranolol, with high concentrations of alprenolol typically used to define non-specific binding at β_3-adrenoceptors and propranolol typically used to define non-specific binding at β_1- and β_2-adrenoceptors (Dunigan *et al.*, 2000).

Competition experiments were performed at the high-affinity site using a range of concentrations of unlabeled drug (β_1-adrenoceptor antagonist CGP20712A, β_2-adrenoceptor antagonist ICI118551 or β_3-adrenoceptor antagonist SR59230A) and 100 pM of [^{3}H]-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 β-counter (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Wells that were not incubated with radiolabel were used for protein determination (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard.

cAMP accumulation assay

Astrocytes were cultured in 96-well plates at a density of 1 × 10^4 cells·per well for 7 days. Cells were serum-starved overnight before the cAMP assay was carried out as previously described (Sato *et al.*, 2008). In each experiment, 100 μM forskolin was used as a positive control and all data expressed as % of this response. To investigate the effect of G_i, cells were treated with *Pertussis* toxin (PTX; 100 ng·mL^{-1}) 16–20 h prior to stimulation with drugs. In experiments investigating the effects of antagonists or weak G_s coupling of β_3-adrenoceptors, cells were treated with 300 nM antagonist or 10 μM forskolin, respectively, added simultaneously with the agonist and incubated for 30 min.

Reverse transcription polymerase chain reaction (RT-PCR)

Astrocytes were cultured for 7 days in six-well plates at a density of 6 × 10^5 cells per well. RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen Australia, Melbourne, Vic., Australia). RNA was also extracted from brown fat and brain from a 3-week-old male FVB mouse. 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(dT_{15}) as previously described (Roberts *et al.*, 1999).

The PCR amplification cycle number and annealing temperatures for each β-adrenoceptor subtype were: β_1- and β_3-adrenoceptors 32 cycles at 64°C, β_2-adrenoceptor 27 cycles at 60°C, β_3-adrenoceptor splice variants 28 cycles at 64°C; performed on cDNA equivalent of 100 ng starting RNA using primers for β_1-, β_2- or β_3-adrenoceptors (Invitrogen; Table 1). For all β-adrenoceptor PCR, PCR mixes contained 0.5 U Platinum Pfx DNA polymerase (Invitrogen), 1 × Pfx buffer, 1 × enhancer solution, 130 μM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM $MgSO_4$, 5.8 pmol forward and 5.8 pmol reverse primer. Following amplification, PCR products were separated by electrophoresis on 1.3% agarose gels and images captured digitally.

[^{3}H]-2-deoxy-D-glucose uptake assay

Astrocytes were grown in 24-well plates at a density of 4 × 10^4 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 [^{3}H]-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 [^{3}H]-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

	Primer (oligonucleotide)	Expected product size
β-Actin	Forward 5′-ATCCTGCGTCTGGACCTGGCTG-3′	441 bp
	Reverse 5′-CCTGCTTGCTGATCCACATCTGCTG-3′	
β_1-Adrenoceptor	Forward 5′-CCGCTGCTACAACGACCCCAAG-3′	414 bp
	Reverse 5′-AGCCAGTTGAAGAAGACGAAGAGGCG-3′	
β_2-Adrenoceptor	Forward 5′-GGTTATCGTCCTGGCCATCGTGTTTG-3′	468 bp
	Reverse 5′-TGGTTCGTGAAGAAGTCACAGCAAGTCTC-3′	
β_3-Adrenoceptor	Forward 5′-GTTGCGAACTGTGGACGTCAGTGG-3′	384 bp
	Reverse 5′-AATGCCGTTGGCGCTTAGCCAC-3′	
β_3-Adrenoceptor (β_{3a}/β_{3b} subtypes)	Forward 5′-CGCGCACCTTCATAGCCATCAAACC-3′	234 bp (β_{3a})
	Reverse 5′-TCTAGTTCCCAGCGGAGTTTTTCATCG-3′	337 bp (β_{3b})

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 K_D and B_{max} (saturation binding experiments) or non-linear curve fitting for one-site competition experiments to obtain pK_i values using the Cheng and Prusoff equation (Cheng and Prusoff, 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^{-4} 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, pK_B values were calculated using the method of Furchgott (1972) where pK_B = log(dose ratio – 1) – log[antagonist]. Glucose uptake experiments were performed in triplicate and data were analysed using non-linear curve fitting (GraphPad PRISM version 5.0) to obtain pEC_{50} values. All results are expressed as mean ± SEM of *n* experiments.

Materials

(±)-CGP20712A (Dr G Anderson, Ciba-Geigy AG, Basel) and zinterol hydroxide (Bristol-Myers Squibb, Noble Park, Vic., Australia) were gifts. Other materials were purchased as follows: [^{3}H] 2-deoxyglucose (specific activity 8.0 Ci·nmol^{-1}) (PerkinElmer Life and Analytical Sciences, Boston, MA, USA); [^{3}H]-CGP12177A (specific activity 50 Ci·nmol^{-1}) (GE Healthcare, Little Chalfont, UK); (±)-ICI118551 (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, Mt Waverly, Vic., Australia); liquid scintillation cocktail (Eco Lite) (MP Biochemicals, Irvine, 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, polyethylenimine (–)-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 β_1-, β_2- and β_3-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. β_1- and β_2-adrenoceptor mRNA were detected in FVB, DBA × C57 and β_3KO astrocytes and β_3-adrenoceptor mRNA was present in FVB, DBA × C57 and $\beta_1\beta_2$KO astrocytes (Figure 1A). This also confirmed that the $\beta_1\beta_2$KO and β_3KO mice were of the correct genotype. The mouse β_3-adrenoceptor gene contains two exons that undergo alternative splicing to produce two splice variants of the mouse β_3-adrenoceptor that are expressed, the β_{3a}-adrenoceptor and the β_{3b}-adrenoceptor (Evans *et al.*, 1999). Using intron-spanning primers that detect both transcripts, it appears that the β_{3a}-adrenoceptor is the predominant isoform in mouse astrocytes (Figure 1B).

[^{3}H]-CGP12177A binding in mouse astrocytes

Receptor binding was used to determine the level of β-adrenoceptor protein expression. A high-affinity $\beta_1\beta_2$-adrenoceptor site was demonstrated in astrocytes from all mouse strains (FVB pK_D 9.57 ± 0.28, B_{max} 96.35 ± 30.59 fmol·mg^{-1} protein, n = 14; β_3KO pK_D 9.79 ± 0.27, B_{max} 89.61 ± 23.09 fmol·mg^{-1} protein, n = 12; DBA × C57 pK_D 9.61 ± 0.17, B_{max} 95.8 ± 41.3 fmol·mg^{-1} protein, n = 5) except $\beta_1\beta_2$KO astrocytes (Figure 2), confirming that the high-affinity

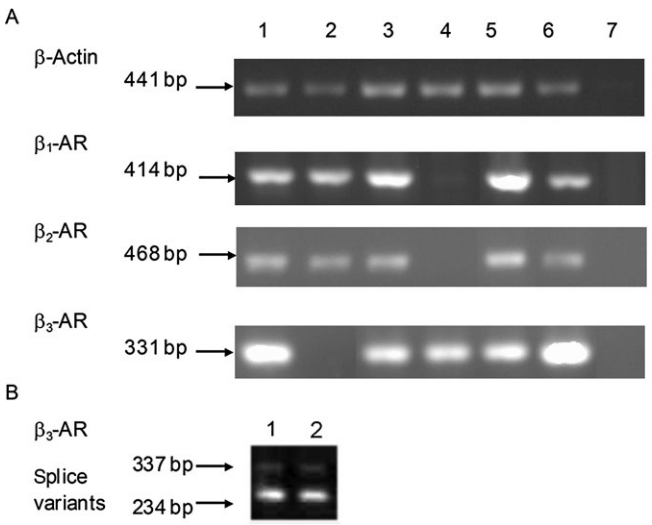

Figure 1

(A) β-Actin, β_1-, β_2- and β_3-adrenoceptor mRNA are expressed in astrocytes isolated from FVB (FVB/N) (Lane 1) and DBA/1 crossed with C57BL/6J (DBA × C57) (Lane 3) mouse strains as well as brain (Lane 5) and brown fat (Lane 6) isolated from an FVB mouse. β_3-Adrenoceptor mRNA was not expressed in β_3-adrenoceptor knockout astrocytes (Lane 2) and β_1- and β_2-adrenoceptor mRNA was not expressed in $\beta_1\beta_2$-adrenoceptor knockout astrocytes (Lane 4). Lane 7 is the negative DNA control. (B) The β_3-adrenoceptor splice variants β_{3a}-adrenoceptor (234 bp) and β_{3b}-adrenoceptor (337 bp) are expressed in FVB (Lane 1) and DBA × C57 (Lane 2) mouse astrocytes. The gels shown are representative of at least three experiments performed. AR, adrenoceptor.

Table 2

Binding affinities for β-adrenoceptor ligands at the low-affinity $\beta_1\beta_2$-adrenoceptor site

Mouse strain	Antagonist	pK_i
FVB/N	CGP20712A	7.81 ± 0.23 (8)
	ICI118551	6.01 ± 0.17 (8)
	SR59230A	6.49 ± 0.23 (9)
β_3-Adrenoceptor knockout	CGP20712A	7.65 ± 0.29 (10)
	ICI118551	6.51 ± 0.18 (9)
	SR59230A	5.99 ± 0.16 (10)
DBA/1 crossed with C57BL/6J	CGP20712A	7.51 ± 0.26 (5)
	ICI118551	6.20 ± 0.20 (5)
	SR59230A	6.19 ± 0.40 (5)

Values are mean pK_i values ± SEM with *n* values in brackets.

site reflects β_1- or β_2-adrenoceptors. Competition for binding at this high-affinity site demonstrated that the selective β_1-adrenoceptor antagonist CGP20712A had the highest affinity whereas affinities of the selective β_2-adrenoceptor antagonist ICI118551 or the β_3-adrenoceptor antagonist SR59230A were much lower (Table 2; Figure 3). This suggests that β_1-adrenoceptors predominate in mouse astrocytes.

β_3-Adrenoceptor saturation experiments demonstrated a low-affinity binding site in astrocytes from all mouse strains *including* β_3KO mice (FVB pK_D 9.12 ± 0.05, B_{max} 224.5 ± 105.9 fmol·mg^{-1} protein, n = 5; β_3KO pK_D 9.15 ± 0.14, B_{max} 303.7 ± 118.7 fmol·mg^{-1} protein, n = 5; DBA × C57 pK_D 9.15 ± 0.02, B_{max} 128.8 ± 66.8 fmol·mg^{-1} protein, n = 3; $\beta_1\beta_2$KO pK_D 9.19 ± 0.32, B_{max} 54.5 ± 13.7 fmol·mg^{-1} protein, n = 3) (Figure 4). The low-affinity binding site in astrocytes from β_3KO mice cannot be due to β_3-adrenoceptors since no mRNA was present (Figure 1), but could be due to [^{3}H]-CGP12177A binding to a low-affinity state of the β_1-adrenoceptor (Konkar *et al.*, 2000).

Effect of the general β-adrenoceptor agonist isoprenaline, the β_2-adrenoceptor agonist zinterol and the selective β_3-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 $\beta_1\beta_2$KO mice (pEC_{50} FVB 8.44 ± 0.4, n = 18; β_3KO 8.64 ± 0.4, n = 15; DBA × C57 8.35 ± 0.3, n = 16; $\beta_1\beta_2$KO not determined, n = 10), suggesting that isoprenaline increases cAMP accumulation by activating β_1- or β_2-adrenoceptors. Zinterol, a β_2-adrenoceptor agonist, increased cAMP accumulation in FVB, β_3KO and DBA × C57 mouse astrocytes, but not in $\beta_1\beta_2$KO astrocytes (pEC_{50} FVB 7.02 ± 0.4, n = 12; β_3KO 7.88 ± 0.5, n = 14; DBA × C57 6.98 ± 0.4, n = 10; $\beta_1\beta_2$KO not determined, n = 10) suggesting that zinterol increases cAMP accumulation by activating β_2-adrenoceptors. The β_3-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 (β_1-adrenoceptor, CGP20712A; β_2-adrenoceptor, ICI118551; β_3-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 $\beta_1\beta_2$KO mice that failed to show significant cAMP accumulation (Table 3; Figure 6). This suggests that β_1-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 β_3-adrenoceptors were weakly coupled to G_s, 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

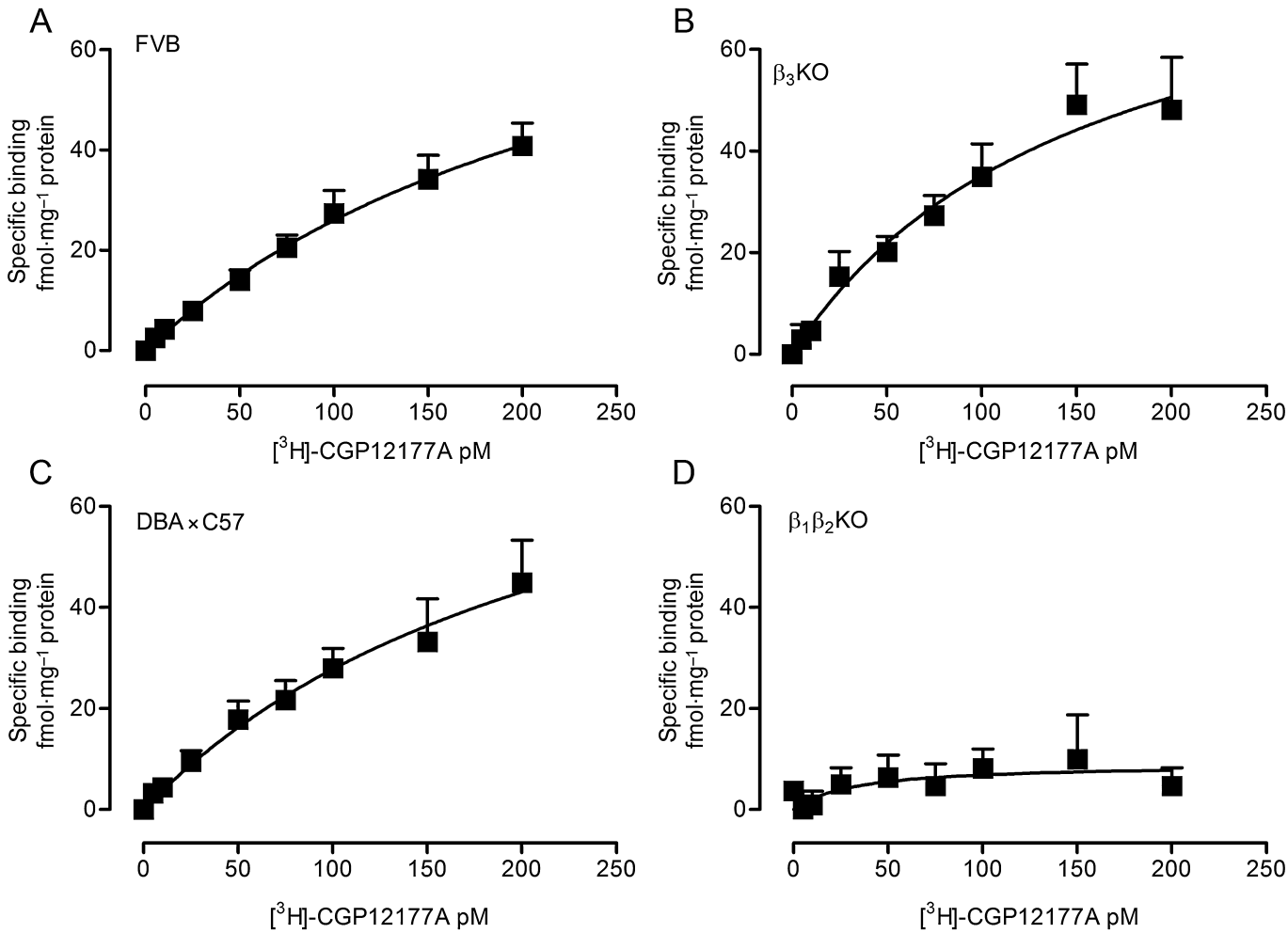

Figure 2

Saturation binding of [^{3}H]-CGP12177A to a high-affinity site in whole astrocytes isolated from (A) FVB (FVB/N) (n = 14), (B) β_3-adrenoceptor knockout (β_3KO) (n = 12), (C) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 5) and (D) $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) (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^{-1} protein. A high-affinity binding site was present in astrocytes from all mouse strains except for the $\beta_1\beta_2$KO mice indicating that the majority of receptors are β_1- or β_2-adrenoceptors at this concentration of [^{3}H]-CGP12177A.

primed with forskolin, cAMP accumulation increased in a concentration-dependent manner [control pEC_{50} not determined; +forskolin pEC_{50} 7.83 ± 0.4, max response (maximum cAMP accumulation achieved expressed as a % of the response to forskolin) 175 ± 15, n = 10]. In β_3KO 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 β_3-adrenoceptors. The DBA × C57 astrocytes showed a similar response to that seen in FVB astrocytes (data not shown). This suggests weak coupling of the β_3-adrenoceptor to G_s.

Effect of the G_i inhibitor PTX on cAMP accumulation in response to isoprenaline in mouse astrocytes

Mammalian β_3-adrenoceptors can couple to both G_s and G_i (Soeder *et al.*, 1999; Hutchinson *et al.*, 2002; 2007; Sato *et al.*, 2005); therefore, the role of G_i was investigated in mouse astrocytes using PTX (100 ng·mL^{-1} overnight) to inhibit G_i. FVB astrocytes treated with PTX demonstrated an enhanced response to isoprenaline compared with isoprenaline alone (control pEC_{50} 8.40 ± 0.9, max response (maximum cAMP accumulation achieved expressed as a % of the response to forskolin) 25.7 ± 3.8; +PTX pEC_{50} 8.43 ± 0.6, max response 41.0 ± 4.4; n = 7, *P < 0.05 two-way ANOVA) (Figure 8). The β_3KO astrocytes showed no significant difference between cells treated with or without PTX (control pEC_{50} 7.93 ± 0.7, max response 20.9 ± 2.4; + PTX pEC_{50} 8.33 ± 0.4, max response 25.5 ± 2.2; n = 4, not significant, two-way ANOVA). DBA × C57 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 $\beta_1\beta_2$KO astrocytes (data not shown).

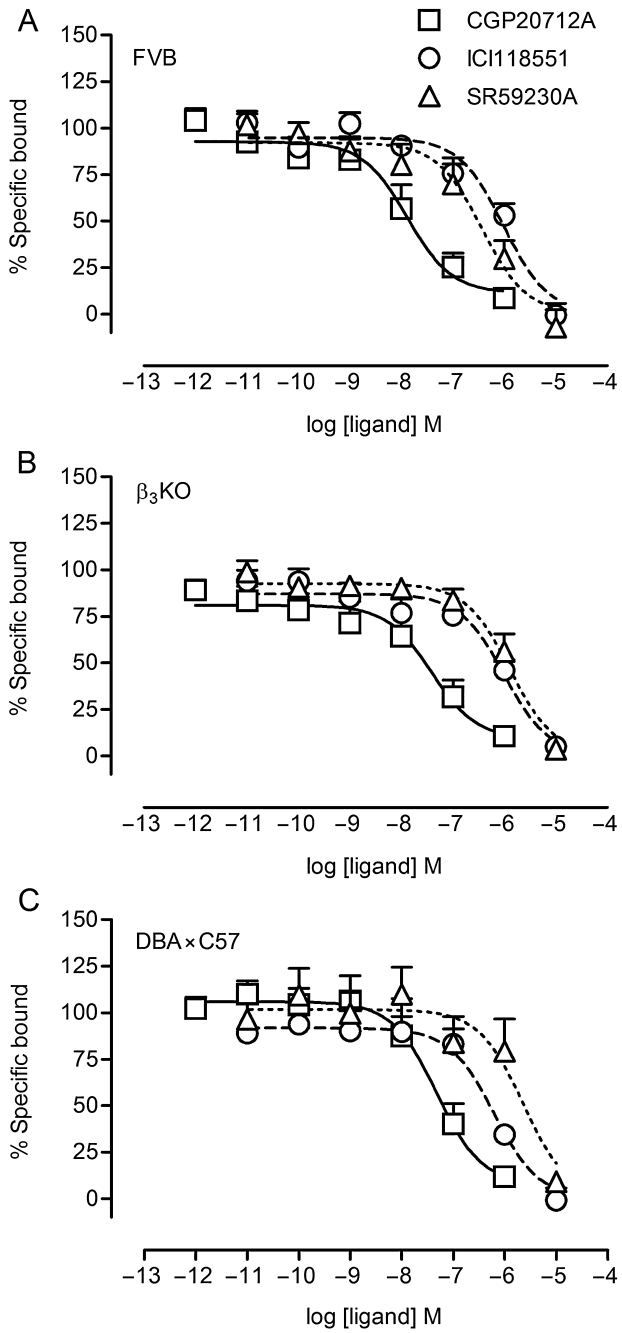

Figure 3

Competition between [^{3}H]-CGP12177A (100 pM) and either CGP20712A (selective β_1-adrenoceptor antagonist), ICI118551 (selective β_2-adrenoceptor antagonist) and SR59230A (selective β_3-adrenoceptor antagonist) for high-affinity binding in whole astrocytes isolated from (A) FVB (FVB/N) (n = 8–9), (B) β_3-adrenoceptor knockout (β_3KO) (n = 9–10) and (C) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 5) mice. Incubations were performed at 37°C for 60 min and non-specific binding defined by (–)-propranolol (1 μM). No significant binding was observed in $\beta_1\beta_2$KO astrocytes (data not shown). The results are expressed as percentage of the maximum specific binding for [^{3}H]-CGP12177A in each individual experiment. Points show mean ± SEM. The dominant population appears to be β_1-adrenoceptors as CGP20712A is able to compete with [^{3}H]-CGP12177A mediated binding most effectively.

Table 3

pK_B values for the selective β-adrenoceptor antagonists, to antagonize isoprenaline-mediated increases in cAMP accumulation in FVB, β_3KO and DBA × C57 astrocytes

Mouse Strain	Antagonist (300 nM)	pK_B
FVB	CGP20712A (β_1-adrenoceptor)	8.61 ± 0.40
	ICI118551 (β_2-adrenoceptor)	7.40 ± 0.20
	SR59230A (β_3-adrenoceptor)	7.40 ± 0.20
β_3KO	CGP20712A	8.33 ± 0.30
	ICI118551	6.82 ± 0.30
	SR59230A	7.39 ± 0.30
DBA × C57	CGP20712A	8.38 ±0.40
	ICI118551	6.92 ± 0.10
	SR59230A	7.54 ± 0.20

Values are mean pK_B values ± SEM of seven experiments performed.
DBA × C57, DBA/1 crossed with C57BL/6J; FVB, FVB/N; β_3KO, β_3-adrenoceptor knockout.

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

The coupling of β_3-adrenoceptors to G_i 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 pEC_{50} 7.17 ± 0.4, max response (maximum cAMP accumulation achieved expressed as a % of the response to forskolin) 224.2 ± 31.5; +forskolin & PTX pEC_{50} 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 (pEC_{50} FVB 9.01 ± 0.23, n = 7; β_3KO 8.79 ± 0.32, n = 7; DBA × C57 8.99 ± 0.29, n = 8; $\beta_1\beta_2$KO 9.22 ± 0.31, n = 9) (Figure 10). Isoprenaline increased glucose uptake in a concentration-dependent manner in all four strains of mouse astrocytes (pEC_{50} FVB 8.88 ± 0.3, n = 18; β_3KO 8.37 ± 0.4, n = 13; DBA × C57 8.89 ± 0.3, n = 16; $\beta_1\beta_2$KO 8.71 ± 0.3, n = 14) (Figure 11). The β_2-adrenoceptor agonist zinterol increased glucose uptake in FVB, β_3KO and DBA × C57 astrocytes but this effect was not present in $\beta_1\beta_2$KO astrocytes (pEC_{50} FVB 9.58 ± 0.2, n = 18; β_3KO 8.99 ± 0.3, n = 13; DBA × C57 9.15 ± 0.3, n = 11; $\beta_1\beta_2$KO not determined, n = 10). The β_3-adrenoceptor agonist

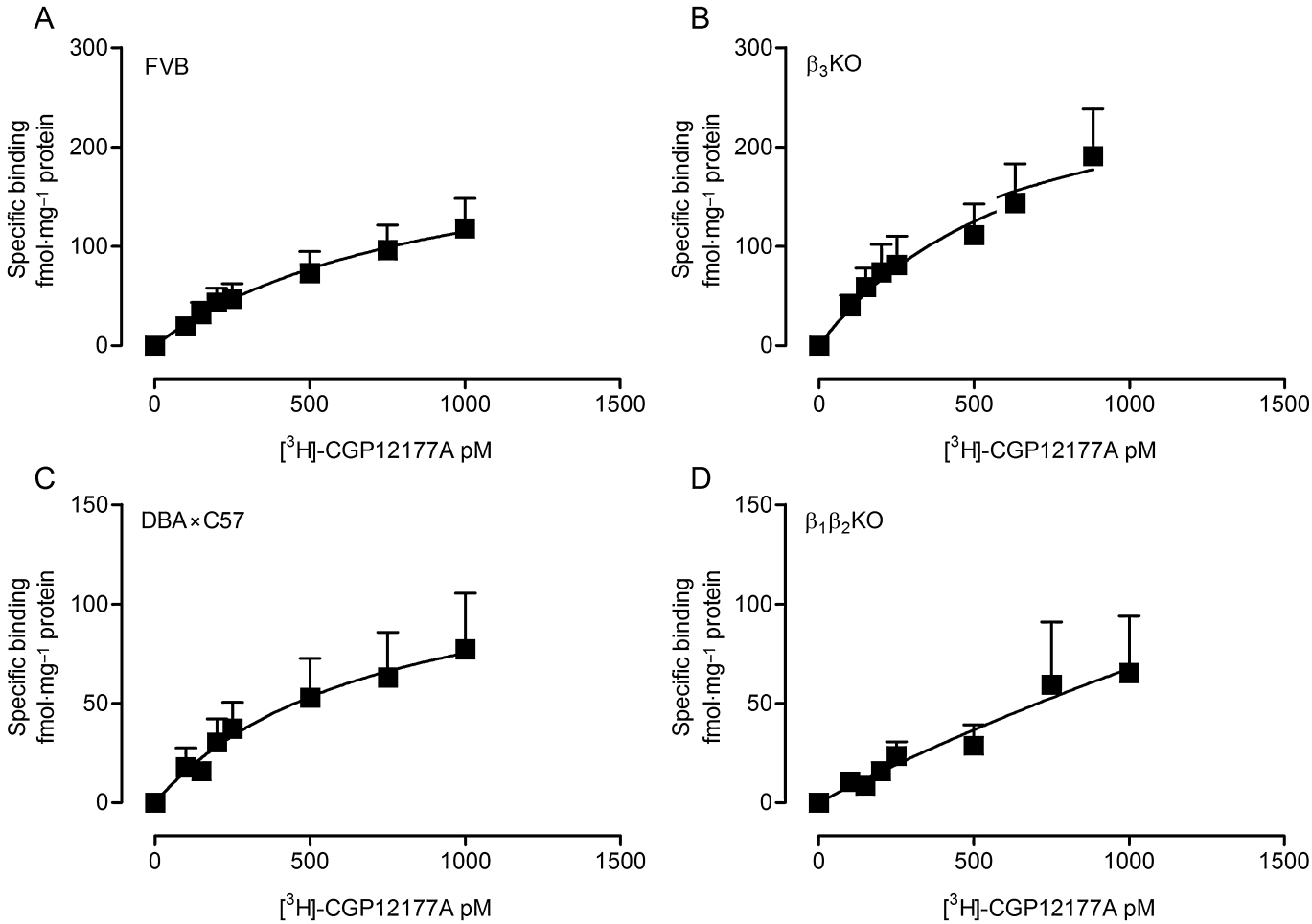

Figure 4

Saturation binding of [^{3}H]-CGP12177A to low-affinity site in whole astrocytes isolated from (A) FVB (FVB/N) ($n = 5$), (B) β_3-adrenoceptor knockout (β_3KO) ($n = 5$), (C) DBA/1 crossed with C57BL/6J (DBA × C57) ($n = 4$) and (D) $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) ($n = 3$) mice. Incubations were performed for 60 min 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.

CL316243 increased glucose uptake in all mouse astrocyte strains except β_3KO (pEC_{50} FVB 9.95 ± 0.4, $n = 20$; β_3KO not determined, $n = 8$; DBA × C57 8.85 ± 0.4, $n = 6$; $\beta_1\beta_2$KO 8.65 ± 0.4, $n = 9$). There was no significant difference in glucose uptake after treatment with isoprenaline or zinterol between FVB and β_3KO astrocytes (two-way ANOVA $P = 0.89$, $P = 0.71$, respectively). Similar results were observed for DBA × C57 and $\beta_1\beta_2$KO astrocytes after treatment with isoprenaline and CL316243 (two-way ANOVA $P = 0.31$, $P = 0.50$, respectively). This suggests that β_2- and β_3-adrenoceptors increase glucose uptake in mouse astrocytes although the role of β_1-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 β_2- and β_3-adrenoceptor protein (Hutchinson *et al.*, 2007). In the present study, β_1- and β_2-adrenoceptor mRNA was identified in astrocytes from all mouse strains tested, except $\beta_1\beta_2$KO; and β_3-adrenoceptor mRNA (including β_{3a}- and β_{3b}-adrenoceptor mRNA) in all strains except for β_3KO. While β_1- and β_2-adrenoceptor mRNA have been demonstrated in rat cultured astrocytes (Joardar *et al.*, 2006; Ghosh and Das, 2007) β_3-adrenoceptor mRNA has not, even

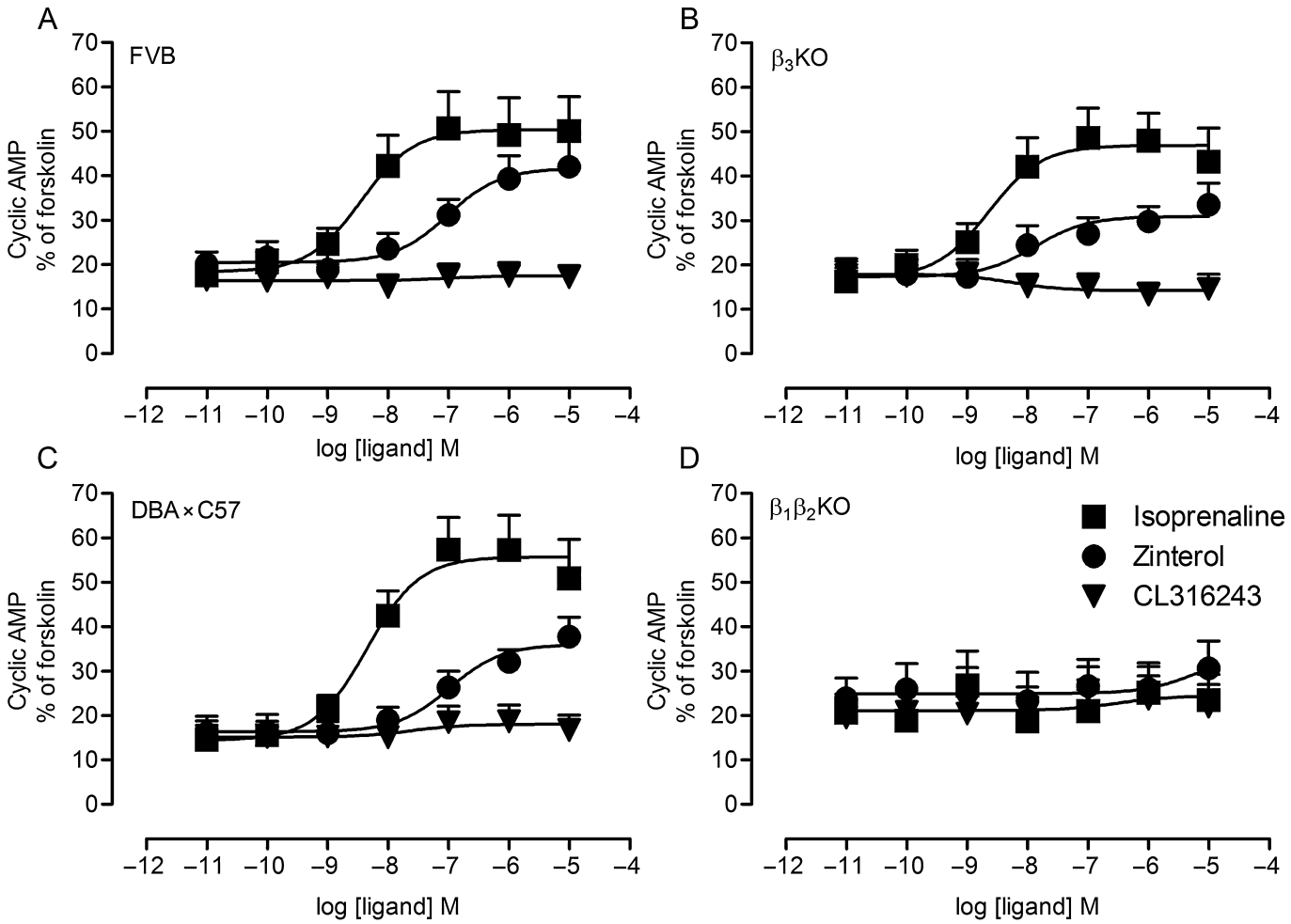

Figure 5

The effect of the β-adrenoceptor agonist isoprenaline, the β_2-adrenoceptor agonist zinterol and the selective β_3-adrenoceptor agonist CL316243 on cAMP accumulation in (A) FVB (FVB/N), (B) β_3-adrenoceptor knockout (β_3KO), (C) DBA/1 crossed with C57BL/6J (DBA × C57) and (D) $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) astrocytes. Results expressed as a % of response to forskolin (10^{-4} M). Each point shows mean ± SEM (n = 10–18).

though it is present in rat (Summers *et al.*, 1995) and mouse brain regions (Evans *et al.*, 1999). The mouse β_3-adrenoceptor occurs as two splice variants, β_{3a}-adrenoceptor and β_{3b}-adrenoceptor (Evans *et al.*, 1999), that share identical pharmacological but different signalling properties (Hutchinson *et al.*, 2002; Sato *et al.*, 2005; 2007; 2008). The β_{3b}-adrenoceptor has higher expression in the brain compared to other tissues (Evans *et al.*, 1999) but in astrocytes, the present study identified both β_{3a}- and β_{3b}-adrenoceptor, with β_{3a}-adrenoceptor predominating.

Radioligand binding studies displayed high-affinity sites in astrocytes from FVB, β_3KO and DBA × C57 mice, but not $\beta_1\beta_2$KO mice. B_{max} 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 β_1-adrenoceptors predominate (Sapena *et al.*, 1996) whereas β_2-adrenoceptors predominate in chick astrocytes (Hutchinson *et al.*, 2007). The β_2-adrenoceptor antagonist ICI118551 also competed for this site, albeit with an affinity intermediate between that expected for β_2- and β_1-adrenoceptors, suggesting that a small population of β_2-adrenoceptors exist in mouse astrocytes. Saturation binding studies also showed a low-affinity site in all mouse strains including β_3KO astrocytes. This is likely to reflect binding of [^{3}H]-CGP12177A to the low-affinity form of the β_1-adrenoceptor (Pak and Fishman, 1996). This compares well to our study where [^{3}H]-CGP12177A only binds to the low-affinity site in astrocytes derived from $\beta_1\beta_2$KO mice. β_3-adrenoceptors cannot be responsible for the low-affinity site shown in β_3KO astrocytes as β_3-adrenoceptor mRNA is not present in these cells, however a low-affinity β_1-adrenoceptor site may account for our results.

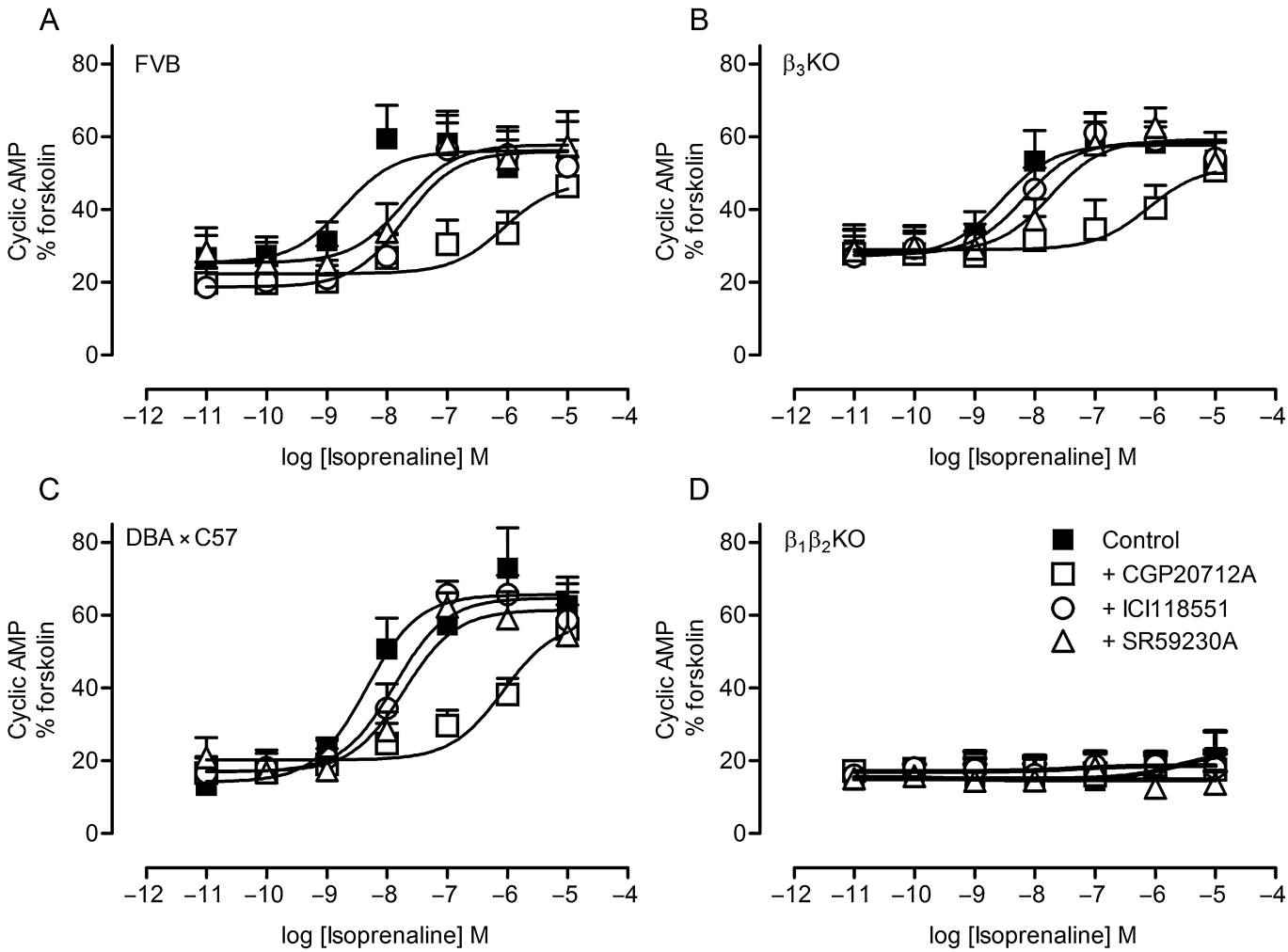

Figure 6

Effect of the selective β_1-adrenoceptor antagonist CGP20712A, the selective β_2-adrenoceptor antagonist ICI118551 and the selective β_3-adrenoceptor antagonist SR59230A in (A) FVB (FVB/N) ($n = 7$), (B) β_3-adrenoceptor knockout (β_3KO) ($n = 7$), (C) DBA/1 crossed with C57BL/6J (DBA × C57) ($n = 7$) and (D) $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) ($n = 9$) 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.

All three β-adrenoceptor subtypes couple to G_s to activate adenylate cyclase and increase intracellular cAMP levels. The non-selective β-adrenoceptor agonist isoprenaline, increased cAMP accumulation in FVB, β_3KO and DBA × C57 astrocytes but not $\beta_1\beta_2$KO astrocytes, indicating that isoprenaline increases cAMP through β_1- or β_2-adrenoceptors. Isoprenaline concentration-response curves were strongly antagonized by CGP20712A with pK_B values (8.3–8.6; Table 3) appropriate for antagonism at β_1-adrenoceptors and not β_2- or β_3-adrenoceptors where pK_B values are significantly lower (Table 4). The β_2-adrenoceptor antagonist ICI118551 weakly antagonized the isoprenaline response (pK_B 6.8–7.4, Table 3), with a pK_B value lower than its known value for β_2-adrenoceptor mediated responses (Table 4). SR59230A, first described as a β_3-adrenoceptor antagonist (Nisoli *et al.*, 1996), antagonized isoprenaline responses with pK_B values of 7.4–7.5 (Table 3), lower than expected for β_3-adrenoceptors that may reflect β_1-adrenoceptor antagonist actions (Table 4) (Manara *et al.*, 1996; Hutchinson *et al.*, 2001). However, in other studies SR59230A (<1 μM) did not affect salbutamol (β_2-adrenoceptor) or dobutamine (β_1-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 β_1-adrenoceptor), but did abolish noradrenaline-mediated effects in mature brown adipocytes (predominately β_3-adrenoceptor) (Nisoli *et al.*, 1996). Because pK_B values for SR59230A were similar in FVB and β_3-adrenoceptor KO astrocytes it is likely that SR59230A antagonizes isoprenaline-mediated cAMP accumulation in these cells by blocking β_1-adrenoceptors. Thus, β_1-adrenoceptors play a greater role than β_2-or β_3-adrenoceptors in isoprenaline-mediated cAMP accumulation in mouse astrocytes. Antagonist pK_B values did not differ between FVB and β_3KO astrocytes (pEC_{50} values

Table 4

Affinity values for β-adrenoceptor antagonists at the β_1-, β_2- or β_3-adrenoceptor

Antagonist	β_1-Adrenoceptor	β_2-Adrenoceptor	β_3-Adrenoceptor
CGP20712A	9.0* (Molenaar and Summers, 1987) 8.1 (Hutchinson *et al.*, 2001)	5.9 (Molenaar and Summers, 1987)	4.8 (Hollenga and Zaagsma, 1989)
ICI118551	7.2 (Bilski *et al.*, 1983)	8.4–9.6 (Nevzorova *et al.*, 2002) 9.3* (Bilski *et al.*, 1983)	5.9–6.0 (Nisoli *et al.*, 1996)
SR59230A	7.4 (Hutchinson *et al.*, 2001) 7.3 (Manara *et al.*, 1996)	6.6 (Manara *et al.*, 1996)	8.2–8.9 (Nisoli *et al.*, 1996) 8.8* (Manara *et al.*, 1996) 7.9–8.2 (Hutchinson *et al.*, 2005) 8.3 (Hutchinson *et al.*, 2001)

*pA_2 values, all other values are pK_B values.

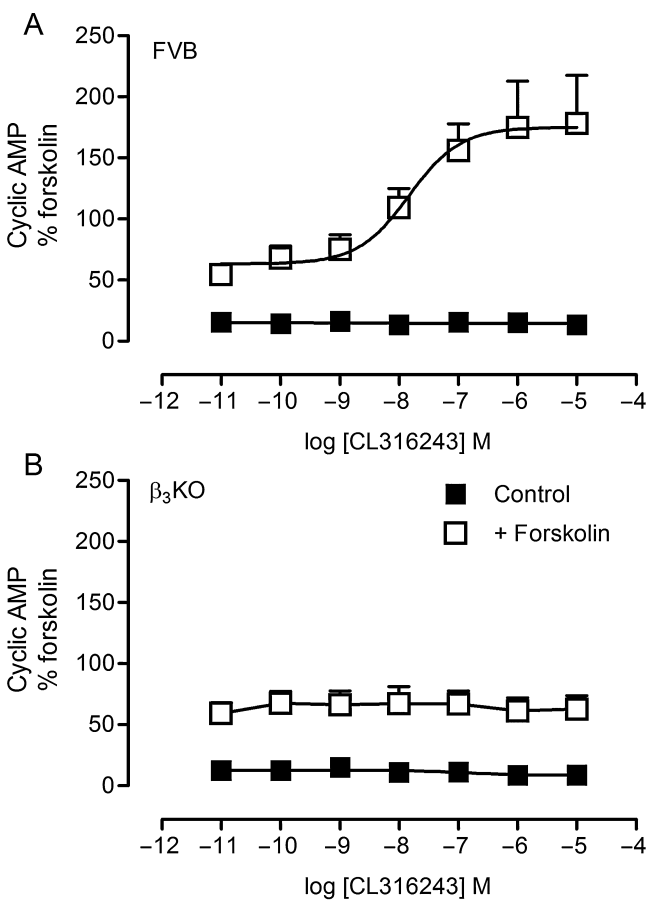

Figure 7

Effect of the selective β_3-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) β_3-adrenoceptor knockout (β_3KO) (n = 10). Results expressed as a percentage of forskolin (10^{-4} M). Each point shows mean ± SEM. Note that in astrocytes containing β_3-adrenoceptors, the response to CL316243 is enhanced in the presence of forskolin.

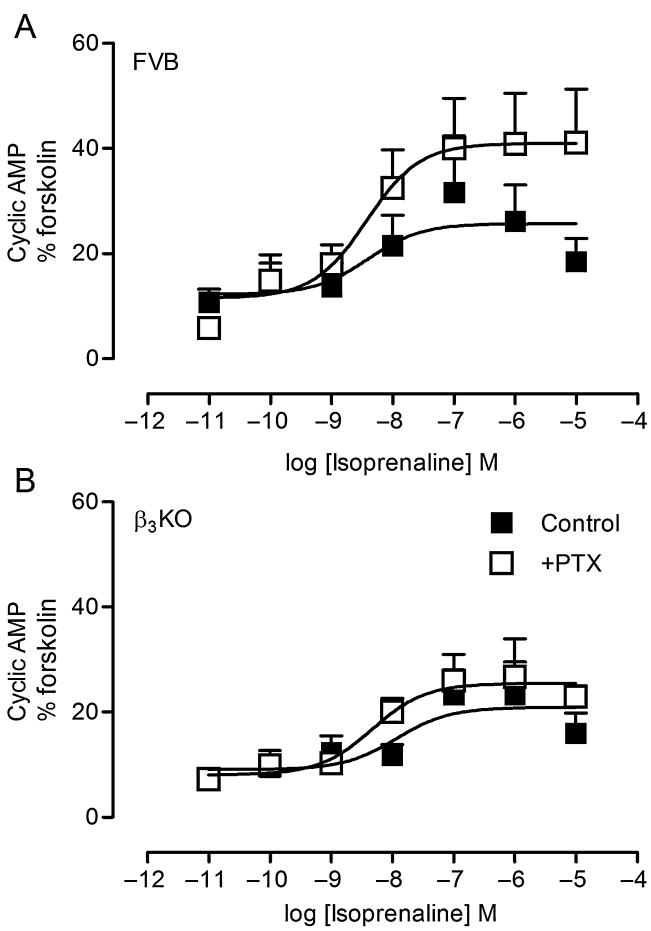

Figure 8

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) β_3-adrenoceptor knockout (β_3KO) (n = 4) mouse astrocytes. Results expressed as a percentage of forskolin (10^{-4} M). Each point shows mean ± SEM.

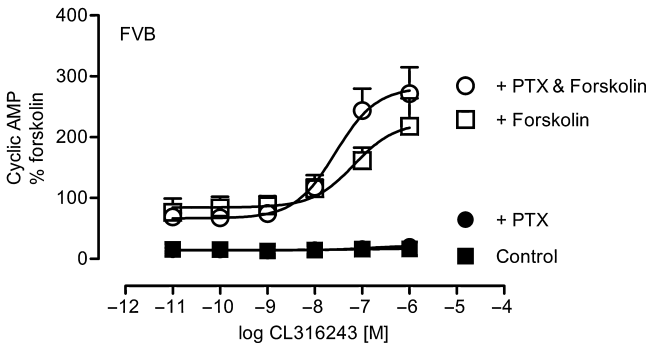

Figure 9

Effect of the selective β_3-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.

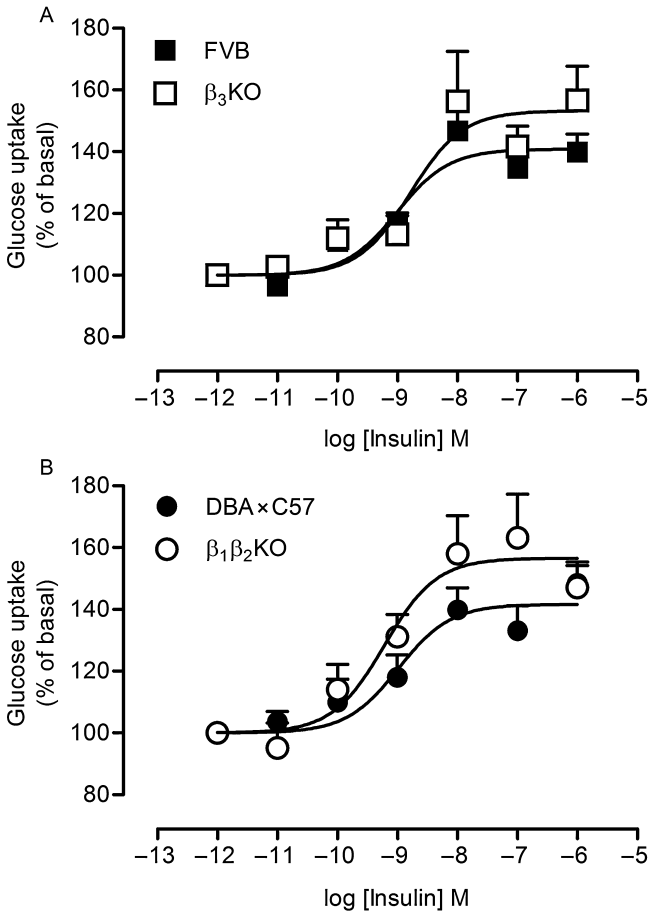

Figure 10

Effect of insulin on [^{3}H] 2-deoxy-D-glucose uptake in whole astrocytes from (A) FVB (FVB/N) (n = 21), β_3-adrenoceptor knockout (β_3KO) (n = 7), (B) DBA/1 crossed with C57BL/6J (DBA × C57) (n = 8) and $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) (n = 9) mice. Results expressed as a percentage of basal and normalized to 100% in each experiment. Each point shows mean ± SEM.

for isoprenaline were also similar) suggesting that β_1-adrenoceptors do not compensate for β_3-adrenoceptors as in ileum from β_3-adrenoceptor KO mice where β_1-adrenoceptor mRNA and protein levels are elevated (Hutchinson *et al.*, 2001). Conversely, in brown adipocytes from β_3-adrenoceptor KO mice, β_1- and α_1-adrenoceptors functionally compensate for β_3-adrenoceptors without altering mRNA or protein levels (Chernogubova *et al.*, 2005). We however saw no compensation in β_3KO astrocytes by β_1- or β_2-adrenoceptors based on mRNA, protein or functional results.

The β_2-adrenoceptor agonist zinterol increased cAMP levels in a concentration-dependent manner in FVB, β_3KO and DBA × C57 astrocytes but not $\beta_1\beta_2$KO astrocytes, indicating that zinterol increases cAMP through β_1- or β_2-adrenoceptors. This is likely to be due to β_2-adrenoceptor stimulation since responses were abolished in $\beta_1\beta_2$KO astrocytes and only one previous study has shown zinterol to act at β_1-adrenoceptors and increase glucose uptake and cAMP levels in brown adipocytes derived from β_3-adrenoceptor KO mice, where β_1-adrenoceptors functionally compensate for lack of β_3-adrenoceptors (Hutchinson *et al.*, 2006). Zinterol can also act as a β_3-adrenoceptor agonist in some circumstances (Hutchinson *et al.*, 2006) although this is not likely here since responses were identical in FVB versus β_3KO astrocytes. This suggests, along with the radioligand binding results, a small population of functional β_2-adrenoceptors in mouse astrocytes.

The β_3-adrenoceptor agonist CL316243 alone was unable to stimulate cAMP accumulation in any of the mouse astrocytes strains used, indicating that β_3-adrenoceptors may not couple strongly enough to G_s in this system to elicit a cAMP response. β_3-Adrenoceptors can also couple to G_i in several systems (Chaudhry *et al.*, 1994; Soeder *et al.*, 1999; Hutchinson *et al.*, 2002; Sato *et al.*, 2005; 2008). Our results show that β_3-adrenoceptors in mouse astrocytes can weakly couple to G_s and G_i based on two different types of experiments. To investigate if β_3-adrenoceptors were G_i coupled, astrocytes were pretreated with the G_i 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 β_3KO astrocytes indicating that β_3-adrenoceptors are coupled to G_i. To investigate if β_3-adrenoceptors can couple weakly to G_s, 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 (pEC_{50} 8.5 ± 0.1). This pEC_{50} value compares well to other studies in cells and tissues expressing moderate-high levels of β_3-adrenoceptors (Hutchinson *et al.*, 2001; 2006; Chernogubova *et al.*, 2005) and suggests that β_3-adrenoceptors are weakly coupled to G_s. CL316243 had no effect on cAMP accumulation in β_3KO mouse astrocytes in the absence or presence of forskolin, confirming this effect is dependent upon the presence of the β_3-adrenoceptor. Assays for cAMP revealed an increased response to CL316243 in the presence of forskolin in $\beta_1\beta_2$KO astrocytes as compared to

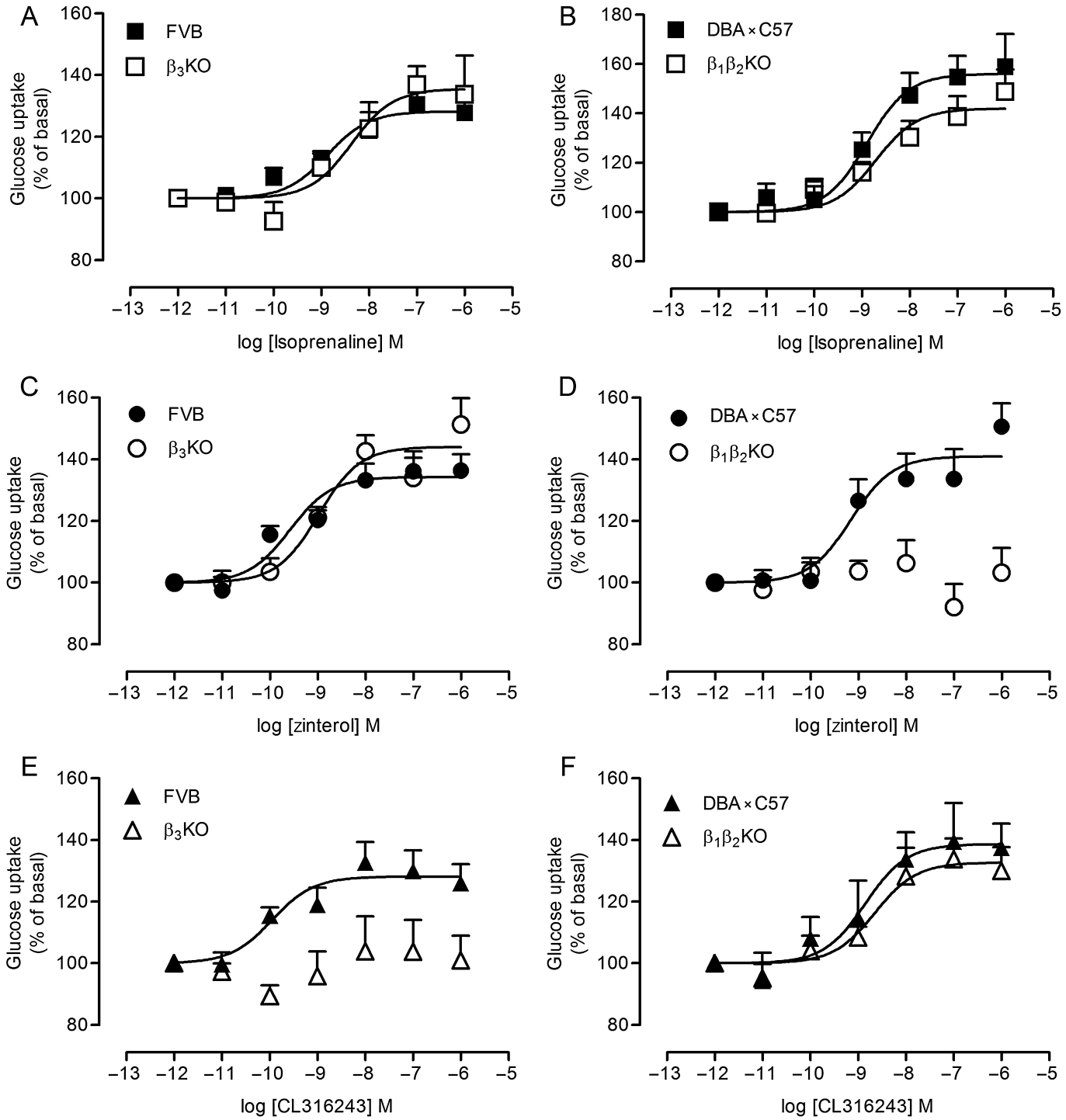

Figure 11

The effect of (A, B) isoprenaline (C, D) zinterol or (E, F) CL316243 on [^{3}H]2-deoxy-D-glucose uptake in whole astrocytes from FVB (FVB/N) (n = 18–20) and β_3-adrenoceptor knockout (β_3KO) (n = 8–13) (A, C, E), and DBA/1 crossed with C57BL/6J (DBA × C57) (n = 6–16) and $\beta_1\beta_2$-adrenoceptor knockout ($\beta_1\beta_2$KO) (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 $\beta_1\beta_2$KO astrocytes and no glucose uptake after treatment with CL316243 in β_3KO astrocytes.

DBA × C57 astrocytes which may indicate a 'supranormal' response to β_3-adrenoceptor agonists as observed by (Rohrer *et al.*, 1999). In $\beta_1\beta_2$KO mice there is a supranormal response to CL316243 in cardiac tissues, which may be due to upregulation of vascular β_3-adrenoceptors in these mice (Rohrer *et al.*, 1999).

Glucose uptake is stimulated by β_2-adrenoceptors in skeletal muscle (Nevzorova *et al.*, 2002) and by β_3-adrenoceptors in brown adipocytes (Chernogubova *et al.*, 2005). β-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 $\beta_1\beta_2$KO astrocytes indicating that it probably acted via β_2-adrenoceptors. The effect cannot be due to actions at β_3-adrenoceptors (Hutchinson *et al.*, 2006) because zinterol responses were intact in β_3KO astrocytes. Additionally CL316243 increased glucose uptake in all astrocyte strains except β_3-KO astrocytes, indicating that β_3-adrenoceptors can also increase glucose uptake. Glucose uptake can follow activation of different intracellular signalling mechanisms on β-adrenoceptor stimulation (Chernogubova *et al.*, 2005; Nevzorova *et al.*, 2006; Hutchinson *et al.*, 2007) which may or not involve coupling to G_s. In chick astrocytes, both β_2- and β_3-adrenoceptors increase glucose uptake (Hutchinson *et al.*, 2007; 2008) with β_2-adrenoceptors increasing glucose uptake over longer periods of time (due to glycogen depletion) through a G_s-mediated mechanism, whereas β_3-adrenoceptors increase glucose uptake rapidly through a G_i-mediated mechanism (Hutchinson *et al.*, 2007). However, the mechanism involved in β-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 β_1- and β_2-adrenoceptors (Ernsberger *et al.*, 1990). Activation of β-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 β-adrenoceptor mRNA subtypes, including β_3-adrenoceptor splice variants, in wildtype mouse astrocytes. β_1-Adrenoceptors are the predominant subtype responsible for the cAMP response with a minor β_2-adrenoceptor component. β_3-Adrenoceptors are coupled to G_i and weakly to G_s whereas β_1- and β_2-adrenoceptors only couple to G_s. This study demonstrates that all β-adrenoceptors are involved in glucose uptake although further investigation is required into the signalling mechanisms involved.

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Conflict of interest

None.

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