Brain, Behavior, and Immunity 56 (2016) 114–129

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Clenbuterol activates the central IL-1 system via the β_2-adrenoceptor without provoking inflammatory response related behaviours in rats

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ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form 18 February 2016
Accepted 25 February 2016
Available online 27 February 2016

Keywords:
β_2-Adrenoceptor
Clenbuterol
Cytokine
IL-1β
Locomotor activity
Food consumption
Sickness behaviour
Anhedonia
Anxiety
Depression

ABSTRACT

The long-acting, highly lipophilic, β_2-adrenoceptor agonist clenbuterol may represent a suitable therapeutic agent for the treatment of neuroinflammation as it drives an anti-inflammatory response within the CNS. However, clenbuterol is also known to increase the expression of IL-1β in the brain, a potent neuromodulator that plays a role in provoking sickness related symptoms including anxiety and depression-related behaviours. Here we demonstrate that, compared to the immunological stimulus lipopolysaccharide (LPS, 250 μg/kg), clenbuterol (0.5 mg/kg) selectively up-regulates expression of the central IL-1 system resulting in a mild stress-like response which is accompanied by a reduction in locomotor activity and food consumption in rats. We provide further evidence that clenbuterol-induced activation of the central IL-1 system occurs in a controlled and selective manner in tandem with its negative regulators IL-1ra and IL-1RII. Furthermore, we demonstrate that peripheral β_2-adrenoceptors mediate the suppression of locomotor activity and food consumption induced by clenbuterol and that these effects are not linked to the central induction of IL-1β. Moreover, despite increasing central IL-1β expression, chronic administration of clenbuterol (0.03 mg/kg; twice daily for 21 days) fails to induce anxiety or depressive-like behaviour in rats in contrast to reports of the ability of exogenously administered IL-1 to induce these symptoms in rodents. Overall, our findings suggest that clenbuterol or other selective β_2-adrenoceptor agonists could have the potential to combat neuroinflammatory or neurodegenerative disorders without inducing unwanted symptoms of depression and anxiety.

1. Introduction

Within the central nervous system (CNS), noradrenaline exerts its anti-inflammatory and neurotrophic effects through actions at glial β_2-adrenoceptors (Day et al., 2014; Mori et al., 2002). Pharmacological stimulation of central β_2-adrenoceptors holds promise as a therapeutic target for the treatment of neuroinflammatory conditions where deficiencies in the central noradrenergic system have been implicated, such as Alzheimer's disease, Multiple Sclerosis and Parkinson's disease (Feinstein et al., 2002; Hertz et al., 2004; Marien et al., 2004). The long-acting brain penetrant β_2-adrenoceptor agonist clenbuterol represents a candidate therapeutic agent for the treatment of neuroinflammation as it shows high affinity for the β_2-adrenoceptor (Liang and Mills, 2001), is highly lipophilic in nature (Smith, 1998) and is resistant to rapid degradation and elimination from the body (Zalko et al., 1998). Clenbuterol is already used in the treatment of respiratory disorders including asthma and chronic obstructive pulmonary disease (Baronti et al., 1980; Boner et al., 1988; Papiris et al., 1986). In addition, evidence indicates that clenbuterol stimulates central glial β_2-adrenoceptors, induces production of neurotrophic factors, and promotes anti-inflammatory and neuroprotective effects (Culmsee et al., 1999a,b; Gleeson et al., 2010; Junker et al., 2002; McNamee et al., 2010a,b,c; Semkova et al., 1996; Teng et al., 2006; Zhu et al., 1999).

We have recently demonstrated that clenbuterol increases expression of the inflammatory cytokine interleukin-1β (IL-1β) in the CNS (McNamee et al., 2010a; Ryan et al., 2011). IL-1β has previously been implicated as a key mediator of neuroinflammation (Allan et al., 2005; Griffin et al., 2006). While low levels of IL-1β play a number of physiological roles in the CNS (Goshen et al., 2007; Pinteaux et al., 2009; Spulber et al., 2009) excessive

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http://dx.doi.org/10.1016/j.bbi.2016.02.023
0889-1591/

production of IL-1β, particularly as a result of microglial activation, has been implicated in the neurodegenerative process (Allan et al., 2005; Basu et al., 2004) and also in the pathogenesis of psychiatric disorders such as depression and anxiety (Connor et al., 1998; Goshen et al., 2008; Koo and Duman, 2008). IL-1β is a potent neuromodulator which can promote the expression of nitric oxide, the prostaglandin synthase cyclooxygenase-II (COX-II) and other pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Dinarello, 1996). This in turn can have detrimental consequences for the CNS (Aschner, 1998; Benveniste et al., 1995; Hofman et al., 1989; Patrizio, 2004; Scheller and Rose-John, 2006; Tizard, 2008; Tracey, 2002). However, it must be noted that we have previously reported that the clenbuterol-induced increase in IL-1β occurs in the absence of an increase in other inflammatory cytokines such as TNF-α, IL-6 and IFN-γ (McNamee et al., 2010a; Ryan et al., 2011).

IL-1β signals by binding to the IL-1 type I receptor (IL-1RI) (Sims et al., 1988). This receptor is present in the CNS (Lynch and Lynch, 2002; McNamee et al., 2010a; Parnet et al., 1994; Pinteaux et al., 2002) but we have previously reported its expression to be unchanged by clenbuterol administration (McNamee et al., 2010a). The actions of IL-1β are regulated by IL-1 receptor antagonist (IL-1ra), which prevents IL-1β from acting on IL-1RI (Carter et al., 1990), and by the IL-1 type II receptor (IL-1RII), a decoy receptor that serves to sequester IL-1 (Colotta et al., 1994), both of which are also expressed in the brain (Pinteaux et al., 2002, 2006). IL-1ra and IL-1RII are thought to prevent excessive or prolonged activation of the IL-1 system (Arend, 2002; Bessis et al., 2000), thus it is noteworthy that increased expression of both occurs in tandem with increased expression of IL-1β following acute clenbuterol treatment in rat cortex (McNamee et al., 2010a). Ultimately, this may serve to moderate the deleterious effects of excessive IL-1β on brain function.

IL-1β, TNF-α, IL-6 and COX-II stand out as being the major players in the induction of sickness behaviour, the stereotypical behavioural response of an animal to injury or infection (Hart, 1988) and the actions of cytokines on the brain (Dantzer, 2001; Dantzer et al., 2008; Hart, 1988). Thus, since clenbuterol is known to induce the expression of IL-1β in the CNS (McNamee et al., 2010a; Ryan et al., 2011), in study 1 we set out to confirm the hypothesis that, as previously reported (McNamee et al., 2010a), clenbuterol induces a selective increase in the IL-1 system in the brain, focusing here on hippocampus and hypothalamus. Therefore, in this study we investigated the expression of IL-β and other pro-inflammatory mediators, namely TNF-α, IL-6 and COX-II following acute administration of clenbuterol. In addition, we measured the expression of the IL-1β receptors IL-1RI, IL-1RII, the antagonist IL-1ra and the downstream signalling molecule inducible nitric oxide synthase (iNOS). For comparative purposes systemic administration of the classical inflammagen lipopolysaccharide (LPS) was also employed since LPS is known to potently induce the expression of these pro-inflammatory mediators in the CNS several hours after administration (Connor et al., 2008; Konsman et al., 2008; O'Sullivan et al., 2009). In tandem to gain insight into the relative activation of glial cells we examined the expression of the astrocyte and microglial activation markers glial fibrillary acid protein (GFAP) and CD11b. As IL-1β is known to have profound effects on behaviour in study 2 we investigated the impact of clenbuterol on several physiological parameters, including body-weight, feeding, locomotor activity, cardiac function and body-temperature, since these have all been implicated in sickness behaviour (Porter et al., 1998; Tizard, 2008; Zhou and Jones, 1993). Notably, clenbuterol has previously been shown to induce behavioural effects in rodents including suppression of locomotor activity and feeding (Geyer and Frampton, 1988; Goldschmidt et al., 1984; O'Donnell, 1993), however, the data are conflicting and the mechanisms underlying these behavioural actions of clenbuterol remain to be elucidated. We hypothesise that the induction of central IL-1β by clenbuterol could mediate the suppressive behavioural effects of clenbuterol. However, as β-adrenoceptors are expressed on many cell types within both the peripheral and central nervous system (Civantos Calzada and Aleixandre de Artinano, 2001; Hein, 2006; Hertz et al., 2004; Mori et al., 2002; Tanaka et al., 2002; Xiang and Kobilka, 2003) in study 3 and 4 we investigated the relative contribution of peripheral versus central β-adrenoceptors in the behavioural actions of clenbuterol in order to gain a better insight.

Neuroinflammatory and neurodegenerative disease states are chronic in nature and require ongoing administration of therapeutics. Therefore, in study 5 we sought to determine the lowest dose of clenbuterol capable of impacting on immune processes in the CNS which also has minimum peripheral effects that could be administered over a prolonged period. Considering the evidence that excessive production of IL-1β is implicated in the neurodegenerative process (Allan et al., 2005) and can precipitate symptoms of depression and anxiety (Koo and Duman, 2008) in study 6 we investigated the ability of chronic treatment with clenbuterol to activate the central IL-1 system and to determine if clenbuterol induces anxiety or depression-like behaviour in rats.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories, UK (weight on arrival: 150–250 g). Rats were maintained in an ambient temperature of 22 ± 2 °C with lighting controlled on a 12 h light-dark cycle (lights on: 08:00 h, lights off: 20:00 h). Food and water were available *ad libitum*. The experimental procedures employed were in compliance with the European Council Directive (86/609/EEC). All animals were handled for at least four days prior to experimentation to alleviate any stress caused by experimental handling or injection.

2.2. Drugs

Clenbuterol and ICI 118,551 were obtained from Tocris Bioscience, UK. LPS from *Escherichia coli* serotype 0111:B4, dexamethasone, propranolol, nadolol and metoprolol were obtained from Sigma-Aldrich, Ireland. All drugs were dissolved in saline (0.9% (w/v) NaCl) and administered via the intraperitoneal (i.p.) or subcutaneous (s.c.) route in an injection volume of 1 ml/kg, with the exception of dexamethasone, which was dissolved in saline containing Tween 20 (2% (v/v)). Saline was administered as a control. As both s.c. and i.p. are parenteral routes of injection which have similar absorption profiles (Turner et al., 2011) we adopt the s.c. route as a suitable alternative to the i.p. route when including telemetrically determined parameters. Radiotelemetry devices were implanted into the abdomen of the rat as previously described (Harkin et al., 2002b). As the heart leads run from the implanted device through two small holes in the abdominal wall to the chest muscle to monitor heart rate (Harkin et al., 2002b) it is not desirable to inject the animals i.p. for risk of disturbing these leads.

2.3. Experimental design

2.3.1. Study 1 – Comparison of the neuroinflammatory response elicited by both LPS & clenbuterol

Rats (n = 6–7) received an i.p. injection of either control, clenbuterol (0.5 mg/kg) or LPS (250 μg/kg) and were euthanized 4 h

post-treatment. The brain was removed from the skull, large blood vessels removed and a portion of hippocampus and hypothalamus were dissected out and placed in RNAlater (Ambion, UK) to ensure integrity of RNA for gene expression analysis. Samples were stored at −80 °C until mRNA expression analysis was performed.

2.3.2. Study 2 – Assessment of behavioural responses to LPS & clenbuterol using telemetry

Rats ($n = 6$) implanted with radiotelemetry devices (see Section 2.4.1) received an s.c. injection of either control, clenbuterol (0.5 mg/kg) or LPS (250 μg/kg). Heart rate, body temperature and locomotor activity of rats were monitored at 10 min intervals for 24 h. Changes in body-weight and food consumption were also measured over the 24 h period.

2.3.3. Study 3: Investigation of the effect of pre-treatment with the synthetic glucocorticoid dexamethasone, the peripherally acting β-adrenoceptor antagonist nadolol or the centrally acting β-adrenoceptor antagonist propranolol on clenbuterol-induced behaviour and IL-1β expression in the brain

A) Rats were treated with either control containing Tween-20 or dexamethasone (1 mg/kg; i.p.) 1 h prior to control or clenbuterol (0.5 mg/kg; i.p.; $n = 5$). B) Rats were treated with either control or nadolol (5 mg/kg; i.p.) 30 min prior to control or clenbuterol ($n = 6$). C) Rats were treated with either control or propranolol (10 mg/kg; i.p.) 30 min prior to control or clenbuterol ($n = 5$). Locomotor activity was measured 2 h post-treatment for 15 min. Following assessment of locomotor activity rats were returned to their home cage and euthanized 4 h post-clenbuterol treatment. The brain was dissected and tissue stored as detailed in Section 2.3.1.

Separate groups of rats were housed individually and treated as above. Food consumption was measured 24 h post-treatment. (A: $n = 5$; B: $n = 5$–6; C: $n = 5$).

A dose of 1 mg/kg dexamethasone was chosen here as we have previously demonstrated that this dose inhibits clenbuterol-induced central IL-1β expression without altering its ability to induce the anti-inflammatory mediators IL-1ra and IL-1RII (Ryan et al., 2011). Rats were pre-treated with dexamethasone to ascertain the impact of blocking clenbuterol-induced IL-1β expression on locomotor activity and food consumption without affecting its anti-inflammatory actions. A dose of 5 mg/kg nadolol was chosen as this has previously been shown to block atropine-induced tachycardia in rats (Hicks et al., 1998). A dose of 10 mg/kg propranolol was used as this was previously shown by our group to block the induction of central IL-1β (McNamee et al., 2010b).

2.3.4. Study 4: Effect of pre-treatment with the selective β_1-adrenoceptor antagonist metoprolol and the selective β_2-adrenoceptor antagonist ICI 118,551 on clenbuterol-induced behavioural changes and IL-1β expression in the brain

Rats were treated with either control, ICI 118,551 (10 mg/kg; i.p.) or metoprolol (10 mg/kg; i.p.) 30 min prior to control or clenbuterol (0.5 mg/kg; i.p.) as previously described (Ryan et al., 2013) and locomotor activity was measured 2 h post-treatment for 15 min. Rats were then returned to their home cage and euthanized 4 h post-clenbuterol treatment. The brain was dissected and tissue stored as detailed in Section 2.3.1 ($n = 5$–6).

A separate group of rats ($n = 6$) implanted with radiotelemetry devices (see Section 2.4.1) was treated as above and heart rate was subsequently monitored at 10 min intervals for 24 h.

The dose of ICI 118,551 used for the purposes of this study was previously shown to significantly reduce clenbuterol-induced apoptosis in the heart (Burniston et al., 2005) while the dose of metoprolol was double the dose used previously by Zhang et al. (2010) to attenuate the clenbuterol-induced increase in heart rate.

2.3.5. Study 5: Dose related effects of clenbuterol

Clenbuterol (0.5 mg/kg) has profound peripheral effects including elevated heart rate, reductions in core temperature and reduced locomotor activity. Thus, we subsequently carried out dose response experiments over a lower range of doses to determine the lowest dose at which clenbuterol can elicit an anti-inflammatory response in rat CNS with minimal peripheral effects which would be more suited for repeated treatment. In the first instance rats were treated with either vehicle or clenbuterol over a range of doses (0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg) and subsequently (0.003 mg/kg, 0.01 mg/kg and 0.03 mg/kg; i.p.) and locomotor activity was measured 2 h post-treatment for 15 min. Rats were returned to their home cage and euthanized 4 h post-clenbuterol treatment for determination of the expression of IL-1 signalling and glial markers as detailed in Section 2.3.1. Separate groups of rats were housed individually and treated as above and food consumption and body weight gain were measured over 24 h following clenbuterol administration. Data from both dose response experiments were combined ($n = 4$–14).

2.3.6. Study 6: Effect of chronic low-dose clenbuterol on anxiety or depression-related behaviour (anhedonia) and IL-1β expression in the brain

Rats ($n = 7$–8) were treated with either control or low-dose clenbuterol (0.03 mg/kg; i.p; twice per day (b.i.d)) at 8 am and 8 pm for 21 days (Fig. 1). This dose of 0.03 mg/kg was selected based on results from study 5 in which it was the lowest dose shown to impact on the IL-1 system and behaviour in addition to being a more therapeutically relevant dose in humans (Daubert et al., 2007). To test for anhedonia, a two-bottle saccharin preference test was carried out for the duration of the study (see Section 2.4.3) whereby the rats had free access to both water and saccharin (0.01%). Rats were tested in the elevated plus maze (see Section 2.4.4) on day 9 and in the open field (see Section 2.4.5) on day 14 to assess anxiety-related behaviours. Rats were euthanized on day 21, 4 h after the final injection. The brain was dissected and tissue stored as described in Section 2.3.1.

2.4. Behavioural measures

2.4.1. Telemetry procedure

Battery-free radiotelemetry devices (PDT 4000HR E-Mitters, Mini Mitter, OR, USA) were implanted into the peritoneal cavity as previously described (Harkin et al., 2002b). Implantation of such devices allows for the acquisition of data for heart rate, core body-temperature and locomotor activity continuously reducing stress associated with exposure of the animal to human contact. Heart rate, body temperature and locomotor activity of rats were monitored at 10 min intervals for 24 h using PDT 4000HR E-mitters and the Windows PC-based acquisition system VitalView™ (Mini-Mitter, USA).

2.4.2. Measurement of locomotor activity

For the studies outlined in Sections 2.3.3–2.3.5 above, rats were removed from their home cage 2 h post-clenbuterol treatment and placed individually into activity monitor cages (32 cm × 20 cm × 18 cm; length × width × height) which were connected to the AM1051 data logger (Benwick Electronics) and active time was logged. Each activity monitor cage is equipped with 2 sets of horizontal infrared beams, positioned at 3 cm and 15 cm above the base of the cage. Both sets of beams consist of a 12 beam by 7 beam matrix, forming a grid of 66 × 2.54 cm^2 cells within the cage. An animal was considered inactive if no beam has been broken for 5 s. Locomotor activity measures were recorded for 15 min, following which time the animals were returned to their home cage.

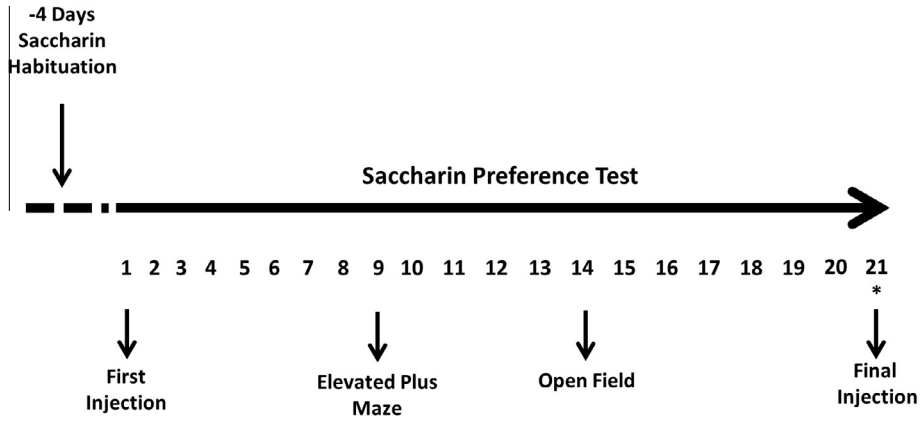

Fig. 1. Chronic clenbuterol administration. Rats were habituated to saccharin exposure for four days prior to the start of the study. Rats were treated with either control or low-dose clenbuterol (0.03 mg/kg; i.p; b.i.d) for 21 days. To assess anhedonia a two-bottle saccharin preference test was carried out for the duration of the study. Rats were tested in the elevated plus maze on day 9 and in the open field on day 14 to assess anxiety-like behaviour. Rats were euthanized on day 21, 4 h after the final injection.

2.4.3. Saccharin preference testing

The saccharin preference test, used as a test for anhedonia, was carried out as previously described (Harkin et al., 2002a). Briefly, animals were exposed to a saccharin solution (0.01%) or tap water in standard drinking bottles in their home cage. The position of the bottles was varied and counter balanced across the left or right side of the feeding compartment. Bottles were weighed daily for 4 days before the commencement of the study. This was carried out to ensure that animals were subdivided into groups with comparable baseline preference. Bottles were subsequently weighed every day post-treatment. Fluid consumption was calculated by measuring the daily difference in bottle weight. Preference scores were calculated by dividing the amount of saccharin consumed by the total amount of fluid consumed (water + saccharin) over the 24 h exposure period. There were 8 rats per treatment group in this study and rats were housed 2 per cage. Cage-mates were in the same treatment group. For saccharin preference measurements the total drinking volumes for each cage of 2 animals was recorded, thus there are 4 pairs per treatment group i.e. $n = 4$.

2.4.4. Elevated plus maze testing

The elevated plus maze was located in a windowless room under dim light conditions. A video camera mounted at ceiling height directly above the open field arena recorded activity during the experimental period. Extra-maze cues (furniture, light source and position of experimenter) were kept constant throughout the study. This test is based on the natural aversion of rodents for open spaces and uses an elevated plus-maze (38.5 cm from the floor) with two open arms and two closed arms ($30 \times 5 \times 15$ cm). Animals were placed individually in the centre of the maze (junction between open and closed arms) and behavioural performance was scored over a period of 5 min. Light intensity was equal in all arms of the maze. The maze was cleaned thoroughly with warm water between trials.

2.4.5. Open field testing

The open field arena was located in a windowless room under dim light conditions. A video camera mounted at ceiling height directly above the open field arena recorded activity during the experimental period. Extra-maze cues (furniture, light source and position of experimenter) were kept constant throughout the study. The open field consisted of a circular arena (120 cm diameter) enclosed by walls (50 cm height). Two zones were defined in the arena: a "safe" (peripheral) zone and an "unsafe" (central) zone. At the start of the task the animal was placed in the centre of the arena and left to explore for a period of 5 min. The following parameters were determined using specialised computer software from EthoVision (Noldus, The Netherlands): (1) time spent in the inner and outer zone; (2) distance travelled in the inner and outer zone; (3) number of entries into the inner zone. The open field arena was thoroughly cleaned between each test.

2.5. Measurement of hypothalamic and hippocampal mRNA expression using qRT-PCR

A Nucleospin RNA II Total RNA isolation kit (Machery-Nagel GmbH & Co.) was used to extract RNA from tissue samples as per the manufacturer's protocol and as previously described (Ryan et al., 2011). The concentration and purity of the RNA samples was determined using a Nanodrop (Thermo Fisher Scientific). A high-capacity cDNA archive kit (Applied Biosystems, UK) was used to reverse transcribe RNA samples according to the manufacturer's protocol. Multi-target (Multiplex) qRT-PCR was carried out using TaqMan gene expression assays containing specific target primers and FAM-labelled MGB target probes (Applied Biosystems, UK) and Taqman® Universal PCR Master Mix on an ABI Prism 7300 instrument (Applied Biosystems) as previously described (Boyle and Connor, 2007). β-actin was used as an endogenous control to normalize gene expression data. Relative gene expression was calculated using the ΔΔCT method (Livak and Schmittgen, 2001) with Applied Biosystems RQ software (Applied Biosystems, UK). Assay IDs are detailed in Supplementary Table 1.

2.6. Statistical analysis of data

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using either a Student's *t*-test or a one- or two-way analysis of variance (ANOVA) followed by a Student Newman-Keuls *post-hoc* test where appropriate. Saccharin preference data were analysed using a two-way ANOVA with repeated measures. A p-value <0.05 was deemed statistically significant.

3. Results

3.1. LPS induces a classical neuroinflammatory response while clenbuterol selectively induces the expression of IL-1 system in rat hippocampus and hypothalamus

One-way ANOVA revealed that there was an effect of treatment on IL-1β mRNA expression (Fig. 2a) in hypothalamus

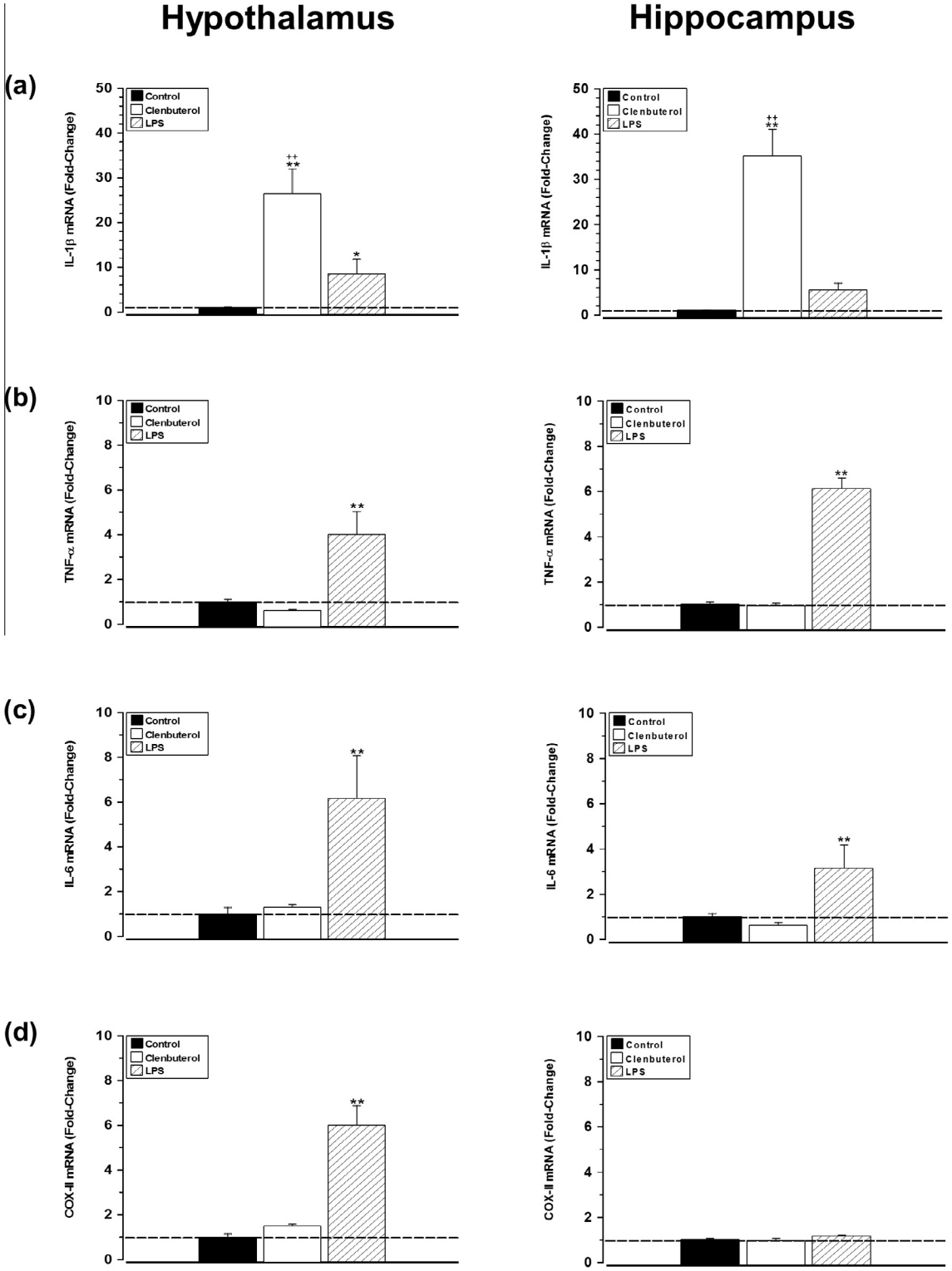

Fig. 2. Clenbuterol selectively induces IL-1β mRNA expression in rat hypothalamus and hippocampus while LPS induces IL-1β, TNF-α, IL-6 and COX-II mRNA expression Clenbuterol (0.5 mg/kg), LPS (250 μg/kg) or control were administered i.p. and rats were sacrificed 4 h post-injection. a) Both LPS and clenbuterol increased the mRNA expression of IL-1β in hypothalamus and hippocampus. b) LPS increased the mRNA expression of TNF-α in hypothalamus and hippocampus while clenbuterol failed to induce TNF-α expression in either brain region. c) LPS increased the mRNA expression of IL-6 in hypothalamus and hippocampus while clenbuterol failed to induce IL-6 expression in either brain region. d) LPS increased the mRNA expression of COX-II in hypothalamus and hippocampus while clenbuterol failed to induce COX-II expression in either brain region. Data are expressed as mean fold-change with standard error of the mean (n = 6–7). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{++}p < 0.01$ versus LPS group (Newman-Keuls *post-hoc* test).

[$F_{(2,17)} = 12.79$, $p < 0.0004$] and hippocampus [$F_{(2,14)} = 26.53$, $p < 0.0001$], TNF-α expression (Fig. 2b) in hypothalamus [$F_{(2,17)} = 11.54$, $p < 0.0007$] and hippocampus [$F_{(2,13)} = 173.40$, $p < 0.0001$], IL-6 expression (Fig. 2c) in hypothalamus

[$F_{(2,17)} = 7.95$, $p < 0.0036$] and hippocampus [$F_{(2,13)} = 7.51$, $p < 0.0068$] and COX-II expression (Fig. 2d) in hypothalamus [$F_{(2,17)} = 33.72$, $p < 0.0001$] but not hippocampus [$F_{(2,13)} = 1.25$, $p = 0.32$] 4 h post-injection. Systemically administered LPS increased IL-1β expression (Fig. 2a) in hypothalamus ($p < 0.05$) but not hippocampus, TNF-α (Fig. 2b) in hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$), IL-6 (Fig. 2c) in hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$) and COX-II (Fig. 2d) in hypothalamus ($p < 0.01$) but not hippocampus 4 h post-injection. In order to examine the induction of IL-1β expression by LPS, the control and LPS groups were isolated and analysed using a Student's *t*-test. This was necessary since the robust clenbuterol-induced increase in IL-1β expression precluded the detection of differences between the control and LPS groups. In contrast, systemically administered clenbuterol selectively induced the expression of IL-1β in both hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$) (Fig. 2a). One-way ANOVA revealed an effect of treatment on IL-1RI in hypothalamus [$F_{(2,17)} = 3.75$, $p = 0.04$] and hippocampus [$F_{(2,13)} = 3.89$, $p = 0.05$], IL-1RII in hypothalamus [$F_{(2,17)} = 127.81$, $p < 0.0001$] and hippocampus [$F_{(2,13)} = 30.62$, $p < 0.0001$], IL-1ra in hypothalamus [$F_{(2,17)} = 5.20$, $p = 0.02$] and hippocampus [$F_{(2,14)} = 21.55$, $p < 0.0001$], iNOS in the hypothalamus [$F_{(2,17)} = 15.84$, $p = 0.0001$] and hippocampus [$F_{(2,14)} = 32.07$, $p < 0.0001$]. LPS increased the expression of IL-1RI ($p < 0.05$), IL-1RII ($p < 0.01$), IL-1ra ($p < 0.05$) in the hypothalamus and iNOS in the hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$) (Table 1). Clenbuterol had a more profound effect than LPS increasing the expression of IL-1RII in the hypothalamus ($p < 0.01$) and hippocampus ($p < 0.01$), IL-1ra in the hypothalamus ($p < 0.05$) and hippocampus ($p < 0.01$) and also on IL-1RI ($p < 0.05$) in the hippocampus but not hypothalamus. Clenbuterol had no effect on iNOS in either brain region. One-way ANOVA also revealed an effect of treatment on GFAP in hypothalamus [$F_{(2,17)} = 3.61$, $p = 0.05$] and hippocampus [$F_{(2,14)} = 21.55$, $p < 0.0001$] and CD11b in hippocampus [$F_{(2,13)} = 4.73$, $p = 0.03$] but not in hypothalamus [$F_{(2,17)} = 2.51$, $p = 0.11$]. *Post-hoc* analyses revealed an increase in the expression of CD11b in the hippocampus ($p < 0.05$) following LPS and clenbuterol. GFAP was increased in the hypothalamus ($p < 0.05$) and hippocampus ($p < 0.01$) following clenbuterol but was not affected by LPS in either brain region.

Table 1
Comparison of clenbuterol- and LPS-induced mRNA changes in hypothalamus and hippocampus. $^{*}p < 0.05$; $^{**}p < 0.01$ versus Control.

	Control	Clenbuterol	LPS
Hypothalamus			
IL-1RI	1 ± 0.12	1.33 ± 0.06	1.60 ± 0.26*
IL-1RII	1 ± 0.09	244.53 ± 16.95**	30.73 ± 11.70**
IL-1ra	1 ± 0.25	2.18 ± 0.11*	2.10 ± 0.47*
iNOS	1 ± 0.17	14.06 ± 3.07	106.96 ± 27.21**
CD11b	1 ± 0.15	1.09 ± 0.13	1.52 ± 0.24
GFAP	1 ± 0.47	8.63 ± 3.39*	2.21 ± 1.34
Hippocampus			
IL-1RI	1 ± 0.08	3.16 ± 0.92*	2.50 ± 0.77
IL-1RII	1 ± 0.32	336.38 ± 42.25**	168.43 ± 53.56
IL-1ra	1 ± 0.14	9.13 ± 2.75**	4.70 ± 0.26
iNOS	1 ± 0.14	10.89 ± 1.58	77.69 ± 17**
CD11b	1 ± 0.12	1.46 ± 0.05*	1.59 ± 0.27*
GFAP	1 ± 0.06	1.59 ± 0.10**	0.91 ± 0.03

3.2. LPS induces sickness behaviour while clenbuterol induces a stress-related response in rats

Locomotor Activity: LPS reduced the locomotor activity of rats, an effect which persisted for approximately 12 h post-injection (Fig. 3a). When locomotor activity data were pooled for the 12 h lights-out period, one-way ANOVA revealed an effect of treatment [$F_{(2,72)} = 20.56$, $p < 0.0001$]. In this regard, LPS reduced locomotor activity compared to controls ($p < 0.01$) and clenbuterol reduced locomotor activity ($p < 0.01$) compared to controls, an effect which persisted for approximately 12 h. When locomotor activity data were pooled for the 12 h lights-out period one-way ANOVA revealed no effect of treatment [$F_{(2,63)} = 1.41$, $p = 0.25$]. *Temperature*: LPS induced a febrile response in rats which persisted for approximately 10 h (Fig. 3b). When temperature data were pooled for the 12 h lights-out period, one-way ANOVA revealed an effect of treatment [$F_{(2,72)} = 107.03$, $p < 0.0001$]. LPS significantly increased body-temperature compared to controls ($p < 0.01$). In contrast, clenbuterol reduced body-temperature of rats, an effect which persisted for approximately 11 h. When temperature data were pooled for the 12 h lights-on period, one-way ANOVA revealed an effect of treatment [$F_{(2,63)}$=47, $p < 0.0001$] clenbuterol decreased body-temperature ($p < 0.01$) compared to controls. *Heart rate*: LPS increased the heart rate of rats, an effect which persisted for approximately 24 h (Fig. 3c). When the heart rate data were pooled for the 12 h lights-out period, one-way ANOVA revealed an effect of treatment [$F_{(2,72)} = 100.61$, $p < 0.0001$]. LPS significantly increased heart rate ($p < 0.01$) compared to controls. Similarly, clenbuterol also increased the heart rate of rats ($p < 0.01$), an effect which again persisted for approximately 24 h, however this was found to be of a much greater magnitude than that observed with LPS. When heart rate data were pooled for the 12 h lights-out period, one-way ANOVA revealed an effect of treatment [$F_{(2,72)} = 100.61$, $p < 0.0001$]. Clenbuterol significantly increased heart rate compared to controls ($p < 0.01$) and also compared to the LPS group ($p < 0.01$). When the heart rate data were pooled for the 12 h lights-on period, one-way ANOVA revealed an effect of treatment [$F_{(2,63)} = 492.12$, $p < 0.0001$]. Clenbuterol again increased heart rate compared to controls ($p < 0.01$) and also compared to the LPS group ($p < 0.01$). *Food consumption & body-weight*: One-way ANOVA also revealed an effect of treatment on food consumption [$F_{(2,15)} = 51.36$, $P < 0.0001$]. Treatment with LPS and clenbuterol both reduced food consumption (Fig. 3d; $p < 0.01$) compared to controls over a 24 h period. One-way ANOVA also revealed an effect of treatment on body-weight gain [$F_{(2,15)}$=7.58, $p = 0.005$] (Fig. 3e). However, while clenbuterol reduced food consumption of rats to a comparable level as that shown with LPS it did not alter the body-weight gain of rats (Fig. 3e).

3.3. The centrally acting β-adrenoceptor antagonist propranolol blocks clenbuterol-induced stress responses and the increase in IL-1β expression in the hypothalamus

Two-way ANOVA revealed an effect of clenbuterol on locomotor activity [$F_{(1,19)} = 427.76$, $p < 0.0001$] but no effect of dexamethasone on locomotor activity [$F_{(1,19)} = 0.002$, $p = 0.97$] (Fig. 4a). Two-way ANOVA revealed a dexamethasone x clenbuterol interaction for food intake [$F_{(1,16)} = 11.73$, $p = 0.004$] but *post-hoc* analyses showed that dexamethasone failed to reverse the clenbuterol-induced reduction in food intake (Fig. 4b). Two-way ANOVA revealed a dexamethasone x clenbuterol interaction for IL-1β [$F_{(1,16)} = 31.15$, $p < 0.0001$]. Clenbuterol induced an increase ($p < 0.01$) in hypothalamic IL-1β expression which was completely blocked by pre-treatment with the synthetic glucocorticoid dexamethasone (Fig. 4c; $p < 0.01$). Two-way ANOVA revealed a nadolol x clenbuterol interaction for locomotor activity [$F_{(1,23)} = 26.13$, $p < 0.0001$] and food intake [$F_{(1,16)} = 57.34$, $p < 0.0001$]. Pre-treatment with the peripherally acting β-adrenoceptor antagonist nadolol inhibited the clenbuterol-induced suppression of locomotor activity (Fig. 4d) and food consumption (Fig. 4e). Two-way ANOVA revealed a nadolol x clenbuterol interaction for IL-1β [$F_{(1,23)} = 14.85$, $p = 0.001$] but *post hoc* analysis showed that nadolol failed to attenuate the clenbuterol-induced increase in IL-1β

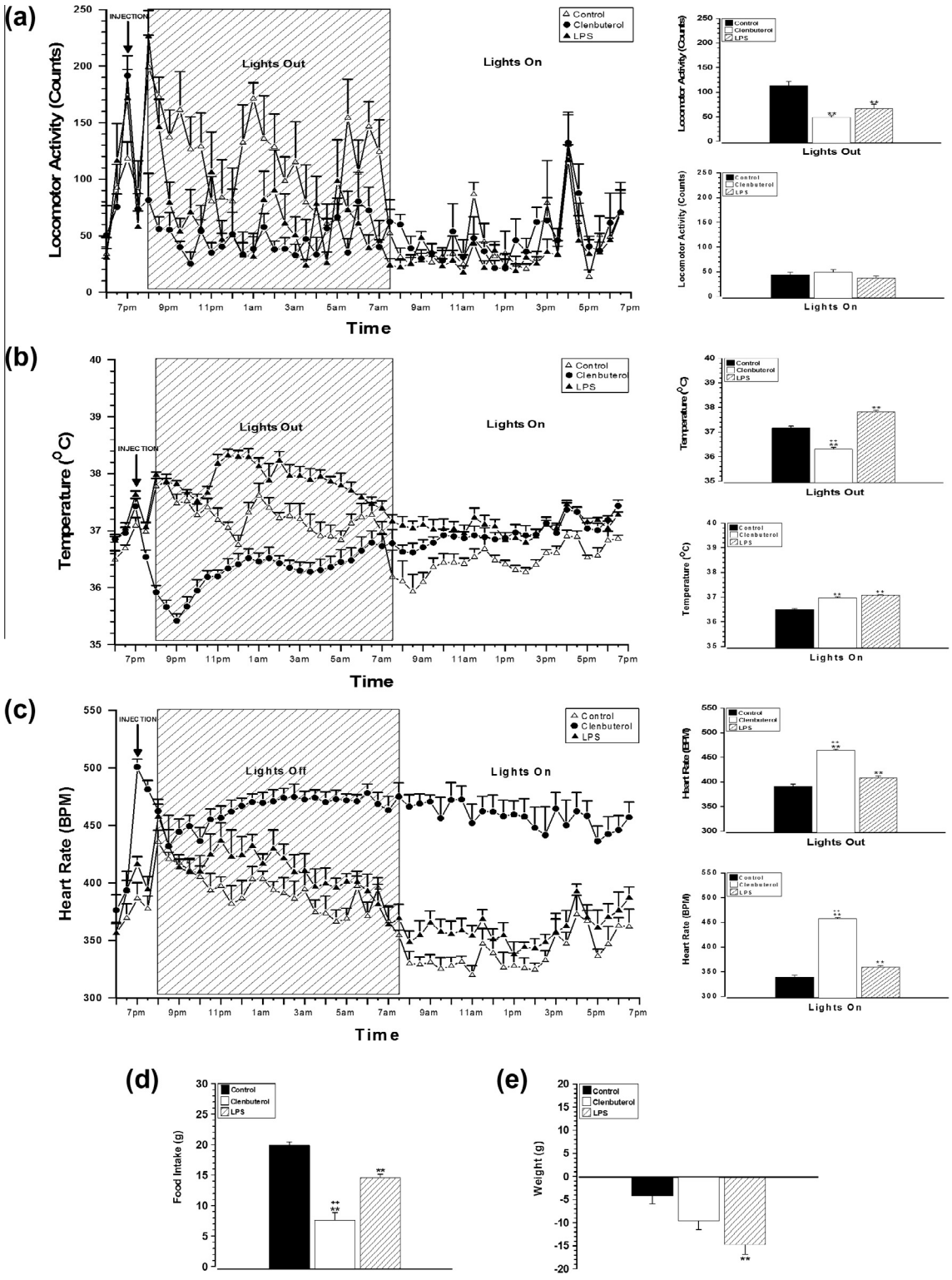

Fig. 3. LPS induces sickness behaviour while clenbuterol induces a stress-related response in rats. Clenbuterol (0.5 mg/kg), LPS (250 μg/kg) or control were administered s.c. to rats and locomotor activity, body-temperature and heart rate were monitored over a 24 h period. a) Both clenbuterol and LPS reduced locomotor activity of rats. When data were pooled for the lights out period both clenbuterol and LPS were found to reduce locomotor activity. b) LPS increases body-temperature while clenbuterol decreases body-temperature of rats. When data were pooled for the lights out period LPS was found to increase body-temperature while clenbuterol was found to reduce body-temperature of rats. c) Both clenbuterol and LPS increased heart rate of rats. When data were pooled for the lights out period both clenbuterol and LPS were found to increase heart rate. d) Clenbuterol and LPS both reduced food intake of rats. e) LPS but not clenbuterol reduced body-weight of rats. Data expressed as means with standard error of the mean ($n = 6$). $^{**}p < 0.01$, $^{*}p < 0.05$ versus control, $^{++}p < 0.01$ versus LPS group (Newman-Keuls *post-hoc* test).

instead further enhancing the clenbuterol-induced increase in hypothalamic IL-1β (Fig. 4f; $p < 0.01$). Two-way ANOVA revealed a propranolol x clenbuterol interaction for locomotor activity [$F_{(1,23)} = 47.38$, $p < 0.0001$] and food intake [$F_{(1,16)} = 10.80$, $p = 0.005$]. Pre-treatment with the centrally acting β-adrenoceptor antagonist propranolol inhibited the clenbuterol-

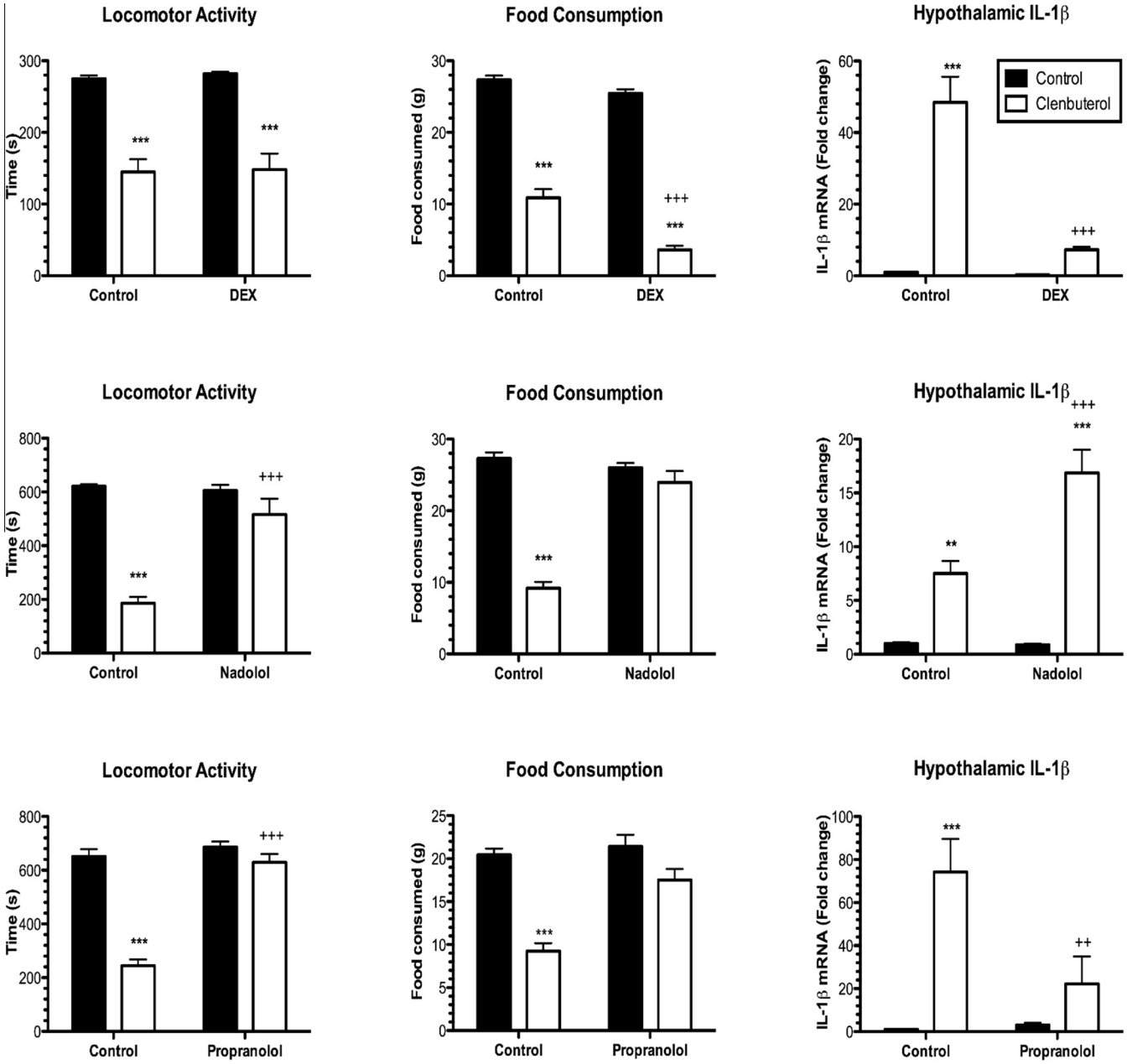

Fig. 4. The centrally acting β-adrenoceptor antagonist propranolol blocks clenbuterol-induced stress responses and the increase in IL-1β expression in the hypothalamus. Rats were pre-treated with dexamethasone (1 mg/kg) 1 h prior to clenbuterol (0.5 mg/kg) treatment or nadolol (5 mg/kg) or propranolol (10 mg/kg) 30 min prior to clenbuterol treatment. Control animals received a saline injection. Locomotor activity was monitored 2 h post-clenbuterol injection. Dexamethasone had no effect on the clenbuterol-induced suppression of locomotor activity (a) and food intake (b) but blocked the clenbuterol-induced increase in hypothalamic IL-1β (c). Nadolol blocked the clenbuterol-induced decrease in locomotor activity (d) and food intake (e) but failed to block the clenbuterol-induced increase in hypothalamic IL-1β (f). Propranolol blocked the clenbuterol-induced decrease in locomotor activity (g) and food intake (h) and attenuated the clenbuterol-induced increase in IL-1β (i). Data expressed as means with standard error of the mean (n = 5–6). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{++}p < 0.01$ versus clenbuterol control counterparts (Newman-Keuls *post-hoc* test).

induced suppression of locomotor activity (Fig. 4g; $p < 0.001$) and food consumption (Fig. 4h). Two-way ANOVA also revealed a propranolol x clenbuterol interaction for IL-1β [$F_{(1,19)} = 13.92$, $p = 0.002$] with propranolol suppressing the clenbuterol-induced increase in hypothalamic IL-1β expression (Fig. 4i; $p < 0.05$).

3.4. The selective β_2-adrenoceptor agonist ICI 118,551 blocks clenbuterol induced hypolocomotion, hypophagia and tachycardia and clenbuterol-induced IL-1β expression in the hypothalamus

One-way ANOVA revealed an effect of treatment on IL-1β mRNA expression in hippocampus [$F_{(3,19)} = 13.52$, $p < 0.0001$] and hypothalamus [$F_{(3,19)} = 29.18$, $p < 0.0001$]. Clenbuterol increased the expression of IL-1β in hypothalamus and hippocampus (Fig. 5a; $p < 0.01$). Pre-treatment with the β_2-adrenoceptor antagonist ICI 118,551 attenuated the clenbuterol-induced increase in IL-1β in both hypothalamus ($p < 0.05$) and hippocampus ($p < 0.01$). Pre-treatment with the β_1-adrenoceptor antagonist metoprolol failed to inhibit the clenbuterol-induced increase in IL-1β. One-way ANOVA revealed an effect of treatment on IL-1RII in hypothalamus [$F_{(3,19)} = 148.53$, $p < 0.0001$] and hippocampus [$F_{(3,19)}$=35.75, $p < 0.0001$], IL-1ra in hypothalamus [$F_{(3,19)} = 44.55$, $p < 0.0001$] and hippocampus [$F_{(3,19)} = 12.65$, $p < 0.0001$], CD11b in hypothalamus [$F_{(3,19)} = 14.27$, $p < 0.0001$) and hippocampus [$F_{(3,19)} = 5.54$, $p = 0.007$] and GFAP in hypothalamus [$F_{(3,19)} = 10.51$, $p = 0.0003$] and hippocampus [$F_{(3,19)} = 12.95$, $p < 0.0001$]. Table 2 shows that

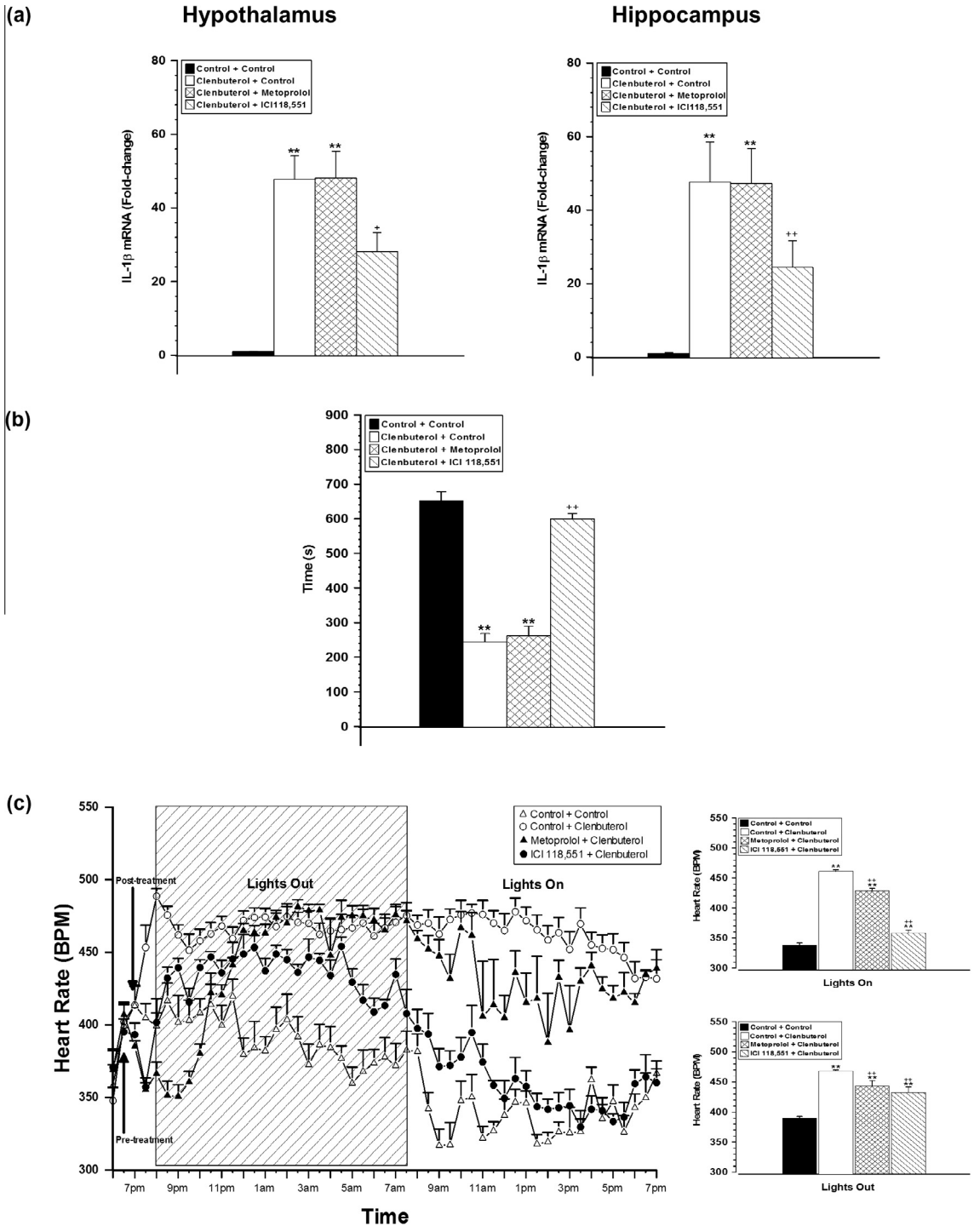

Fig. 5. Clenbuterol-induced central IL-1β expression, hypolocomotion and tachycardia are mediated by the β_2-adrenoceptor. Metoprolol (10 mg/kg), ICI 118, 551 (10 mg/kg) or control were administered 30 min prior to administration of clenbuterol (0.5 mg/kg; i.p.) or control and rats were euthanized 4 h post-clenbuterol injection. a) Clenbuterol increased IL-1β in hypothalamus and hippocampus and this was attenuated by pre-treatment with ICI 118,551 but not metoprolol. b) Clenbuterol suppressed locomotor activity of rats and this effect was blocked by pre-treatment with ICI 118,551 but not metoprolol. c) Clenbuterol increased heart rate over a 24 h period. Pre-treatment with metoprolol or ICI 118,551 attenuated the clenbuterol-induced increase in heart rate. Data expressed as means with standard error of the mean ($n = 6$). $^{**}p < 0.01$ versus control, $^{++}p < 0.01$, $^{+}p < 0.05$ versus clenbuterol control counterparts (Newman-Keuls *post-hoc* test).

ICI 118,551 inhibited the clenbuterol-induced increase in IL-1RII, IL-1ra and CD11b in both hypothalamus and hippocampus and GFAP in the hippocampus. One-way ANOVA revealed an effect of treatment on locomotor activity [$F_{(3,20)} = 68.12$, $p < 0.0001$] and food consumption [$F_{(3,20)} = 57.47$, $p < 0.0001$]. Clenbuterol decreased the time rats were active in the locomotor activity cages (Fig. 5b; $p < 0.01$) compared to controls. ICI 118,551 inhibited the clenbuterol-induced decrease in active time ($p < 0.01$). Clenbuterol decreased food consumption (Table 2; $p < 0.01$) compared to controls. ICI 118,551 inhibited the clenbuterol-induced decrease in food consumption ($p < 0.01$). In contrast, metoprolol had no effect on the clenbuterol-induced increase in molecular markers, decrease in active time or decrease in food consumption. One-way ANOVA revealed an effect of treatment on heart rate

Table 2
Effects of the selective β_2-adrenoceptor antagonist ICI 118, 551 and the selective β_1-adreonceptor antagonist metoprolol on clenbuterol-induced changes in mRNA expression in hypothalamus and hippocampus. $^{*}p < 0.05$; $^{**}p < 0.01$ versus Control + Control. $^{+}p < 0.05$; $^{++}p < 0.01$ versus Clenbuterol + Control.

	Control + Control	Clenbuterol + Control	Clenbuterol + Metoprolol	Clenbuterol + ICI118,551	p-value
Hypothalamus					
IL-1RII	1 ± 0.16	343.78 ± 24.51**	329.93 ± 22.61**	20.37 ± 5.05++	p < 0.0001
IL-1ra	1 ± 0.02	1.91 ± 0.02**	1.94 ± 0.11**	1.57 ± 0.10**,+	p < 0.0001
CD11b	1 ± 0.05	1.53 ± 0.10**	1.56 ± 0.08**	1.13 ± 0.07++	p < 0.0001
GFAP	1 ± 0.12	1.83 ± 0.13**	2.02 ± 0.15**	1.47 ± 0.12*	p < 0.0001
Hippocampus					
IL-1RII	1 ± 0.18	523.58 ± 88.88**	498.59 ± 59.11**	12.79 ± 4.31++	p < 0.0001
IL-1ra	1 ± 0.24	6.12 ± 1.16**	5.87 ± 0.65**	2.39 ± 0.40++	p < 0.0001
CD11b	1 ± 0.07	1.62 ± 0.21**	1.41 ± 0.03*	1.19 ± 0.10+	p = 0.0066
GFAP	1 ± 0.05	1.62 ± 0.14**	1.48 ± 0.06**	1.10 ± 0.09++	p = 0.0002
Locomotor Activity					
Active Time(s)	651.33 ± 27	244.5 ± 23.44**	262.33 ± 27.28**	600.33 ± 15.24++	p < 0.0001
Food Consumption					
Food consumed (g)	24.7 ± 0.59	9.85 ± 1.13**	12.87 ± 1.29**	23.72 ± 0.81++	p < 0.0001

[$F_{(3,96)} = 41.6$, $p < 0.0001$] during the 12 h lights-out period and during the 12 h lights-on period [$F_{(3,88)} = 230.98$, $p < 0.0001$]. Clenbuterol induced tachycardia in rats, an effect which persisted for 24 h post-injection (Fig. 5c). When heart rate data were pooled for the 12 h lights-out period clenbuterol increased heart rate ($p < 0.01$) compared to controls. ICI 118,551 attenuated clenbuterol-induced tachycardia, an effect which persisted for 24 h. When heart rate data were pooled for the 12 h lights-out period ICI 118,551 reduced clenbuterol-induced tachycardia ($p < 0.01$). Notably, during the 12 h lights-on period ICI 118,551 induced a profound reduction in clenbuterol-induced tachycardia ($p < 0.01$). Metoprolol attenuated clenbuterol-induced tachycardia, an effect which lasted approximately 5 h before wearing off. When heart rate data were pooled for the 12 h lights-out period metoprolol induced a reduction in clenbuterol-induced tachycardia ($p < 0.01$).

3.5. Clenbuterol has a dose-dependent effect on IL-1β, IL-1ra and IL-1RII mRNA expression and locomotor and feeding behaviour

Clenbuterol was shown to dose-dependently increase the expression of IL-1β, IL-1RII and IL-1ra in both the hypothalamus and hippocampus (Supplementary Table 2). The lowest dose of clenbuterol found to induce mRNA expression of these markers was 0.03 mg/kg. Clenbuterol also dose-dependently suppressed locomotor activity and food consumption with 0.03 mg/kg the lowest dose found to impact on both of these behaviours (Supplementary Table 2).

3.6. Chronic low dose clenbuterol selectively activates the central IL-1 system and activates astrocytes but not microglia

Chronic clenbuterol increased IL-1β expression in hypothalamus and hippocampus (Fig. 6a; $p < 0.001$). IL-1ra expression was induced in hippocampus (Fig. 6b; $p < 0.01$) but not hypothalamus. Clenbuterol failed to induce expression of IL-1RI (Fig. 6c) but caused a robust increase in the expression of IL-1RII in hypothalamus and hippocampus (Fig. 6d; $p < 0.001$). Importantly, clenbuterol did not induce expression of IL-6 or IL-10 (Table 3). Notably, clenbuterol treatment significantly reduced IL-6 expression compared to controls in the hippocampus (Table 3; $p < 0.01$). Clenbuterol induced an increase in TNF-α expression in the hypothalamus (Table 3; $p < 0.05$) but not the hippocampus. Clenbuterol induced GFAP expression in hypothalamus and hippocampus (Table 3; $p < 0.01$) and CD11b in the hypothalamus (Table 3; $p < 0.01$) but not the hippocampus.

3.7. Chronic low-dose clenbuterol does not induce anxiety or depressive-like behaviour

While acute clenbuterol (0.03 mg/kg; i.p.) decreases locomotor activity of rats (Fig. 7a; $p < 0.01$) chronic low-dose clenbuterol (0.03 mg/kg; i.p.; b.i.d.) does not alter locomotor activity when compared to controls. In the open field test (Fig. 7b) there was no difference between control and clenbuterol-treated groups in terms of number of visits to the inner zone ($p = 0.76$), time spent in the inner zone ($p = 0.38$) or distance travelled in the inner zone ($p = 0.46$). In the elevated plus maze test (Fig. 7c) there was no difference between control and clenbuterol-treated animals in terms of number of entries into open arms ($p = 0.96$) and time spent in the open arms ($p = 0.44$). Two-way ANOVA revealed no significant clenbuterol x time interaction for saccharin preference [$F_{(7,42)}=0.31$, $p = 0.95$] (Fig. 7d). Chronic clenbuterol did not impact on food intake or body-weight (Supplementary Fig. 2).

4. Discussion

The results presented here confirm our previous findings (McNamee et al., 2010a; Ryan et al., 2013) showing that systemic administration of clenbuterol induces a selective induction of the IL-1 system in rat brain. However, while acute treatment with clenbuterol (0.5 mg/kg) has profound peripheral and behavioural effects these do not represent classic sickness behaviour and can be dissociated from induction of the IL-1 system. Instead these effects appear to be linked to the increase in heart rate and the induction of a mild stress-like response. In addition, chronic treatment with clenbuterol, at a therapeutically relevant dose of 0.03 mg/kg, induces expression of the IL-1 system without provoking anxiety or depressive-like behaviours highlighting the potential of using clenbuterol without producing adverse behavioural effects.

A classical neuroinflammatory response and the concomitant behavioural response associated with it are generally accepted to be mediated by the induction and combined actions of the pro-inflammatory and pyrogenic mediators IL-1β, TNF-α, IL-6 and COX-II in the CNS (Beishuizen and Thijs, 2003; Blatteis et al., 2005; Cao et al., 1995; Dantzer et al., 2008; Tizard, 2008). In study 1 we show that, as previously reported (Connor et al., 2008; Konsman et al., 2008; O'Sullivan et al., 2009), systemically administered LPS causes a classical neuroinflammatory response indicated by an increase in the expression of IL-1β, TNF-α, IL-6 and COX-II in the rat brain and is accompanied by classical sickness behaviour (Hart, 1988) characterized by fever, increased heart rate,

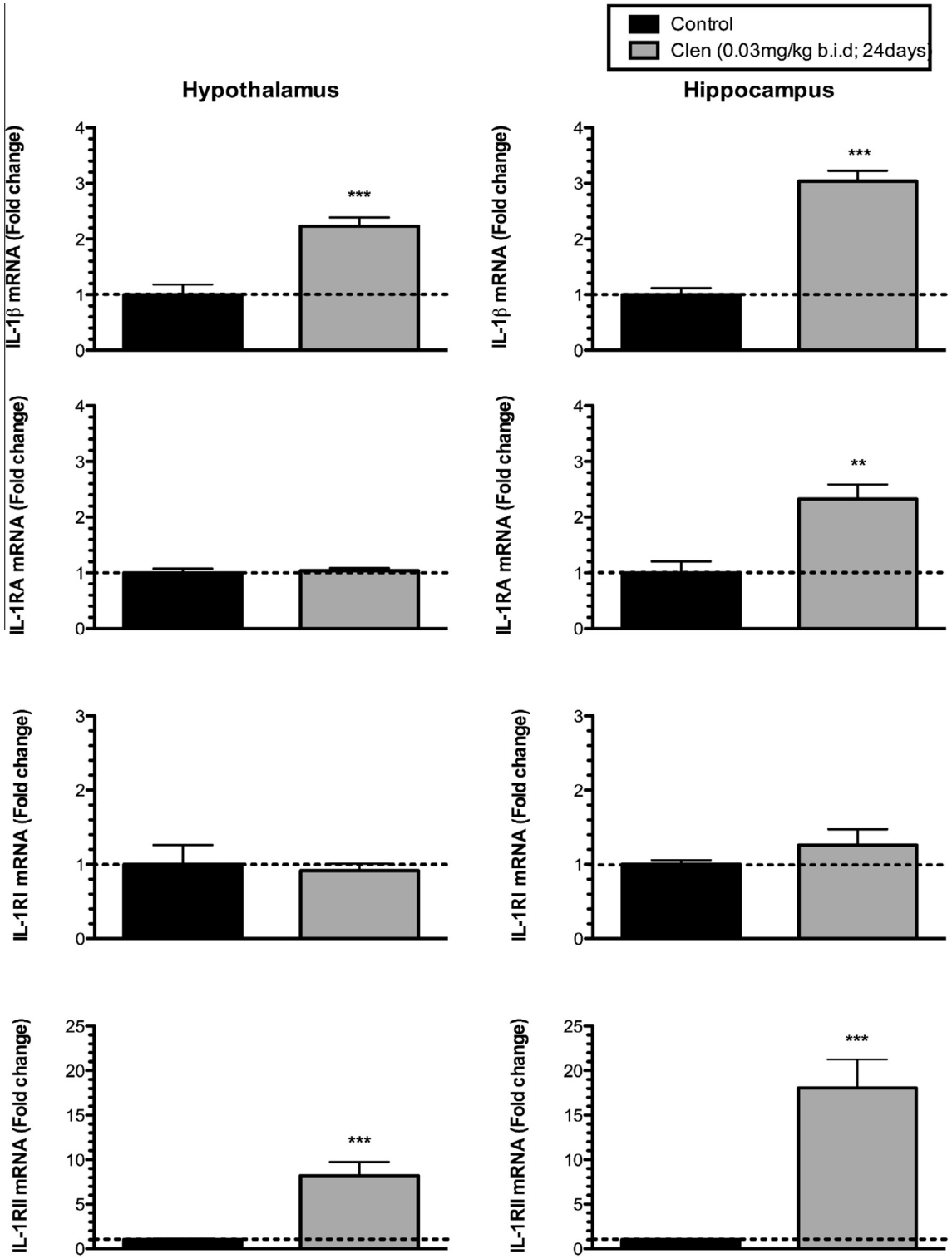

Fig. 6. Chronic low-dose clenbuterol treatment activates the IL-1 system in hypothalamus and hippocampus. Clenbuterol (0.03 mg/kg; b.i.d.) or control was administered i.p. for 21 days. Clenbuterol induced a) IL-1β mRNA expression in hypothalamus ($p < 0.001$) and hippocampus ($p < 0.001$) and b) IL-1ra expression in hippocampus ($p < 0.01$) compared to controls. Clenbuterol had no effect on c) IL-1 Type-I receptor mRNA expression hypothalamus or hippocampus. d) Clenbuterol induced expression of IL-1 Type-II receptor in hypothalamus ($p < 0.001$) and hippocampus ($p < 0.001$) compared to controls. Data expressed as mean fold-change with standard error of the mean ($n = 8$). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control (Student's *t*-test).

hypophagia, weight loss and reduced locomotor activity. In contrast, clenbuterol selectively increases expression of the central IL-1 system in the absence of a classical inflammatory and behavioural response. Notably, the clenbuterol-induced increase in the IL-1 system is more robust than that associated with LPS. Since previous studies have shown that acute administration of clenbuterol has no effect on the expression of the IL-1 Type I receptor or IL-1 accessory protein in rat CNS, both of which are imperative for IL-1 signalling (McNamee et al., 2010a), the expression of iNOS was evaluated as a down-stream measure of IL-1 signalling and we

Table 3
Effects of chronic clenbuterol on mRNA expression in hypothalamus and hippocampus. *p < 0.05; **p < 0.01 versus Control (Student's *t*-test).

	Control	Chronic clenbuterol	p-value
Hypothalamus			
TNF-α	1 ± 0.07	1.23 ± 0.07*	p = 0.0406
IL-6	1 ± 0.02	0.93 ± 0.15	p = 0.2162
IL-10	1 ± 0.14	0.88 ± 0.10	p = 0.4928
CD11b	1 ± 0.05	1.23 ± 0.05**	p = 0.0078
GFAP	1 ± 0.09	1.58 ± 0.15**	p = 0.0050
Hippocampus			
TNF-α	1 ± 0.10	1.26 ± 0.09	p = 0.0722
IL-6	1 ± 0.08	0.68 ± 0.08**	p = 0.0052
IL-10	1 ± 0.19	1.48 ± 0.34	p = 0.3164
CD11b	1 ± 0.09	1.20 ± 0.09	p = 0.1261
GFAP	1 ± 0.07	1.37 ± 0.06**	p = 0.0018

show that clenbuterol does not significantly alter its expression in comparison to LPS. Thus, it appears that, in line with a previous report (Shirato et al., 2007), clenbuterol induces a mild stress response as opposed to sickness behaviour. This we suggest is caused by its profound peripheral effects observed in study 2, in particular its effects on heart rate. Clenbuterol has previously been reported to induce changes in heart rate in both *in vitro* and *in vivo* systems (Hoey et al., 1995; Mazzanti et al., 2007). While the β_1-adrenoceptor is the pre-dominant adrenoreceptor subtype expressed in the heart (Brodde et al., 2006; Wallukat, 2002) and although clenbuterol acts principally on β_2-adrenoceptors, the clenbuterol-induced increase in heart rate is suggested to be mediated by an interplay between both β_1- and β_2-adrenoceptor subtypes (Mazzanti et al., 2007). In line with this, the clenbuterol-induced effect on heart rate in this study was found in study 4 to be significantly attenuated by the selective β_2-adrenoceptor antagonist ICI 118,551 but also to a lesser extent by the selective β_1-adrenoceptor antagonist metoprolol. The dose of metoprolol (10 mg/kg) used here was double the dose of 5 mg/kg which was shown previously by Zhang et al. (2010) to attenuate the clenbuterol-induced increase in heart rate.

As mentioned, clenbuterol induces a profound but selective increase in the expression of the central IL-1 system which has previously been implicated as a key mediator of neuroinflammation (Allan et al., 2005; Griffin et al., 2006). However, there is now a general consensus that inflammatory mediators including IL-1β can exert both adaptive and maladaptive responses in the brain, depending on the concentration, the duration of exposure and overall immune status (Goshen et al., 2007; Pinteaux et al., 2006; Spulber et al., 2009). While there is a body of research outlining the contribution of IL-1β to the neurodegenerative process (Allan et al., 2005), evidence also indicates that IL-1β may elicit neuroprotective effects both *in vitro* and *in vivo* (Mason et al., 2001; Shaftel et al., 2007; Tanabe et al., 2009; Tomozawa et al., 1995). Taken together, these findings suggest that IL-1β can have either protective or detrimental effects in the CNS depending on the context, and that constitutive expression of IL-1β is necessary for many aspects of normal brain function. The clenbuterol-induced increase in IL-1β occurs in tandem with an increase in IL-1ra and IL-1RII which, as previously described (Arend, 2002; Bessis et al., 2000; Colotta et al., 1994), may act to prevent excessive or prolonged activation of the IL-1 system. Moreover, we have previously reported that the clenbuterol-induced increase in IL-1β is accompanied by increased activation of the mitogen-activated protein k inase/extracellular-signal-regulated kinase (MAP kinase/ERK) signalling pathway which is associated with pro-survival effects in neurons but fails to activate c-Jun N-terminal kinase (JNK) and P38 MAP kinase pathways, which have been implicated in mediating the detrimental effects of IL-1 in the brain (McNamee et al., 2010a). The results presented in study 4 show that the clenbuterol-induced increase in IL-1β, and also IL-1ra and the IL-1 Type II receptor, expression is mediated by β_2-adrenoceptors as expression could be blocked by ICI 118,551 but not metoprolol.

It has been demonstrated that astrocytes support hippocampal-dependent memory and long-term potentiation via IL-1 signalling (Ben Menachem-Zidon et al., 2011). Thus, in order to get an indication of the cellular source of clenbuterol-induced IL-1β we examined expression of the astrocytic marker (GFAP) and microglial activation marker (CD11b) and found an increase in GFAP expression in both hypothalamus and hippocampus. Further work is required to clarify the cellular source of clenbuterol-induced IL-1β. In line with previous *in vitro* work (Junker et al., 2002), the increase in GFAP, observed following systemic clenbuterol administration is mediated by β_2-adrenoceptors since in study 4 ICI 118,551 impeded the expression of GFAP in hippocampus and hypothalamus.

Previous reports have shown that peripheral injection of IL-1β results in prolonged increases in brain noradrenaline, an effect demonstrated to reduce the locomotor activity of rats (Wieczorek and Dunn, 2006). We show here that clenbuterol has a more profound impact on locomotor activity than LPS, an effect which may be linked to the sizeable increase in central IL-1β. However, previous studies have shown that the clenbuterol-induced reduction in locomotor activity can be attenuated by administration of hydrophilic non-selective β-adrenoceptor antagonists which are incapable of penetrating the blood brain barrier indicating that the effects of clenbuterol on locomotor activity may be mediated by peripheral and not central mechanisms (Geyer and Frampton, 1988; O'Donnell, 1993). Therefore, we sought to determine if the suppression of locomotor activity induced by clenbuterol was dependent on its ability to induce central IL-1β expression. We have previously demonstrated that pre-treatment with 1 mg/kg of the synthetic glucocorticoid dexamethasone can inhibit clenbuterol-induced central IL-1β expression without altering its ability to induce the anti-inflammatory mediators IL-1ra and IL-1RII suggesting a mechanistic dissociation between the ability of β_2-adrenoceptor activation to induce IL-1β and its negative regulators (Ryan et al., 2011). Thus, in study 3 we pre-treated rats with dexamethasone to ascertain a role for central IL-1β in the clenbuterol-induced suppression of locomotor activity and food consumption. However, while dexamethasone blocked the clenbuterol-induced increase in central IL-1β it failed to inhibit its suppressive effect on locomotor activity and food consumption. Moreover, the peripherally acting β-adrenoceptor antagonist nadolol failed to inhibit clenbuterol-induced central IL-1β expression but completely blocked the suppressive effect on locomotor activity and food consumption. Notably, treatment with propranolol, a brain penetrable non-selective β-adrenoceptor antagonist, blocked both the induction of central IL-1β and suppression of locomotor activity and food consumption. Taken together these data suggest that peripheral β-adrenoceptors mediate the suppression of locomotor activity and food consumption induced by clenbuterol and that this occurs independently of its ability to induce central IL-1β expression. However, this is not surprising as previous studies indicate that peripheral β-adrenoceptors mediate the suppressive effect of clenbuterol on behaviour (Geyer and Frampton, 1988; O'Donnell, 1993). Moreover, we suggest that the clenbuterol-induced behavioural effects are mediated by the impact of clenbuterol on cardiac β_2-adrenoceptors and the increase in heart rate since the effects were completely blocked when rats were pre-treated with ICI 118,551 but not metoprolol. This same study showed that clenbuterol reduced food intake and this effect was reversed when the clenbuterol-induced tachycardia was attenuated by ICI 118,551. Thus while exogenous IL-1β is known

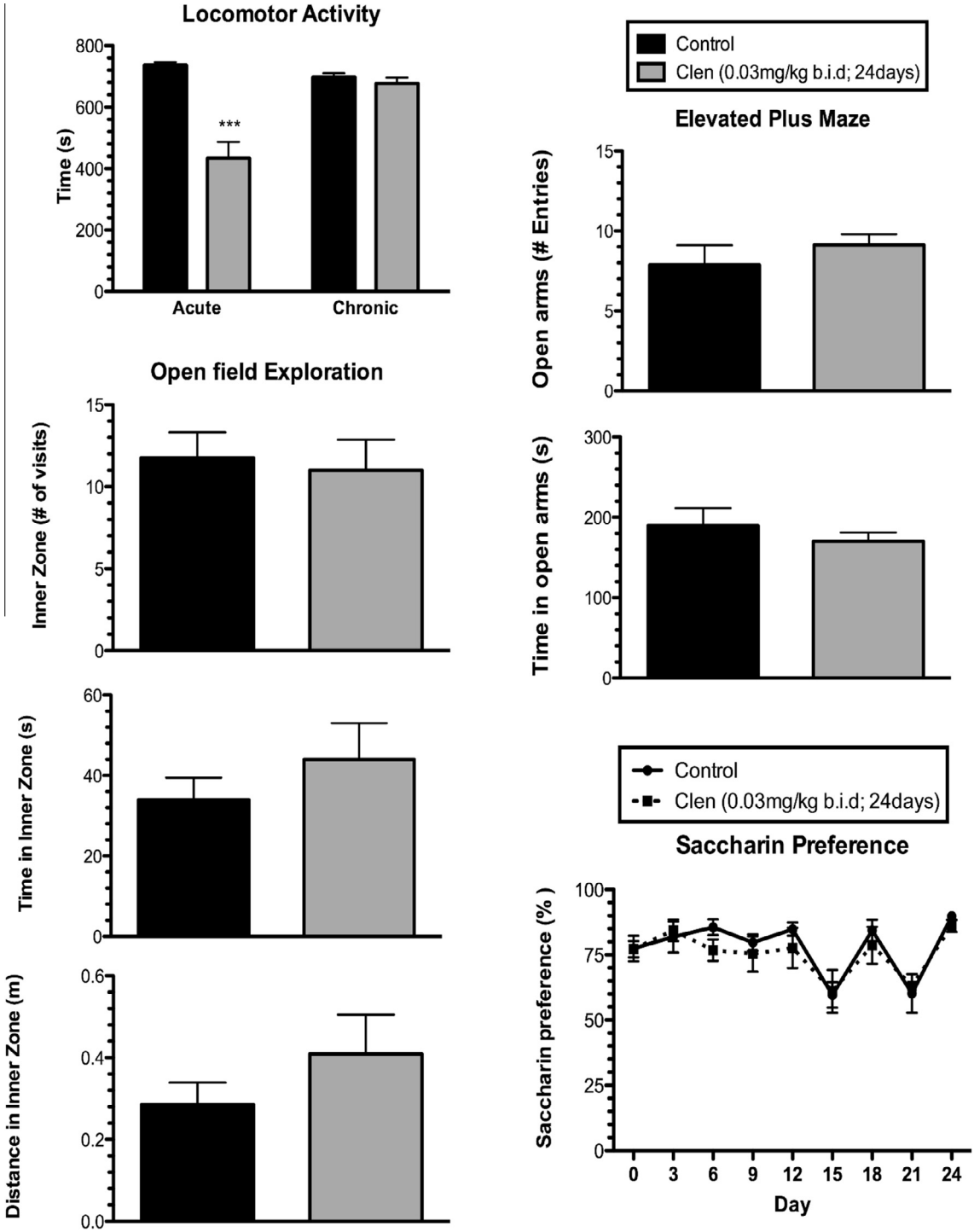

Fig. 7. Chronic low-dose clenbuterol does not induce anxiety or depressive-like behaviours. Clenbuterol (0.03 mg/kg; b.i.d.) or control were administered i.p. for 21 days. a) Acute clenbuterol significantly reduced locomotor activity ($p < 0.001$) compared to control counterparts but chronic clenbuterol did not impact on locomotor activity. Chronic clenbuterol did not alter behaviour in b) the open field test with respect to number of visits to the inner zone, time spent in the inner zone, distance travelled in the inner zone as assessed by Student's *t*-test. Chronic clenbuterol did not alter behaviour in c) the elevated plus maze with respect to number of entries into open arms or time spent in open arms as assessed by Student's *t*-test. d) Chronic clenbuterol did not alter saccharin preference over the 21 day experimental period as assessed by a two-way repeated-measures ANOVA. Data expressed as means with standard error of the mean (a–c: $n = 8$; d: $n = 4$). ***$p < 0.001$ versus control (Student's *t*-test).

to induce behavioural changes including suppression of locomotor activity and feeding (Bluthe et al., 1996; Kent et al., 1992, 1994; Plata-Salaman et al., 1988; Swiergiel and Dunn, 2000, 2007), the results of the present study clearly show that the induction of central IL-1β by clenbuterol does not play a role in the behavioural effects elicited by this drug.

Study 5 identified that the lowest dose of clenbuterol capable of impacting on immune processes in the CNS was 0.03 mg/kg, therefore this dose was chosen to be used for chronic treatment in study 6 to determine if its central and peripheral effects persist over time. Notably, this dose is in line with that already used therapeutically in humans (Daubert et al., 2007). A dose of 10–20 μg

(0.01–0.02 mg) is efficaciously used in humans for bronchodilation over prolonged periods up to twice or three times daily (Hida et al., 1985; Kamburoff et al., 1977; Wheatley, 1982). While we show that this low-dose of clenbuterol increases heart rate following acute administration, its effects are transient and persist for approximately 10 h before returning to baseline (Supplementary Fig. 1). This is noteworthy since clenbuterol toxicity in humans is known to lead to symptoms including headache, dizziness, nervousness, peripheral vasodilation, hypothermia and tachycardia, however, previous reports indicate that clenbuterol causes an increase in blood flow and heart rate during the initial days of treatment but these effects are found to subside with continued treatment (Hoey et al., 1995). We show that chronic treatment with low-dose clenbuterol induces a 2.5–3 fold increase in IL-1β mRNA expression compared to a 15–40 fold increase observed following acute administration of the same dose. Thus, IL-1β expression subsides following continued clenbuterol administration in hypothalamus and hippocampus. As before, we found that chronic clenbuterol increased expression of the anti-inflammatory molecules IL-1ra and IL-1RII.

Considering the evidence that exogenous IL-1β can precipitate symptoms of depression and anxiety we sought to investigate the effect of chronic treatment with clenbuterol on anxiety and depression-like behaviours (anhedonia) in rats and show that chronic clenbuterol does not induce either depression- or anxiety-like behaviours. In addition, chronic clenbuterol had no effect on body weight or food intake which suggests that the reduction in feeding seen after acute clenbuterol administration is transient and the animals regain their appetite and maintain a healthy weight with continued treatment. There is now a general consensus that inflammatory mediators including IL-1β can exert both adaptive and maladaptive responses in the brain, depending on the concentration, the duration of exposure and the overall immune environment (Goshen et al., 2007; Pinteaux et al., 2006; Spulber et al., 2009). The fact that clenbuterol-induced IL-1β does not induce an anxious or depressed phenotype following a prolonged period of treatment could be partly due to the low concentration of IL-1β expressed or to the induction of IL-1ra and the IL-1RII.

It is well documented that stimulation of β-adrenoceptors either directly or through increased release of noradrenaline in response to various stressors can induce expression of pro-inflammatory cytokine, in particular IL-1β, both peripherally and centrally (Blandino et al., 2006, 2009; Johnson et al., 2005, 2004). Moreover, blocking noradrenaline reuptake facilitates increases in IL-1β both peripherally and centrally under both basal and stress-induced conditions (Blandino et al., 2006). Stress has also been found to increase the expression of IL-1RII in the brain in tandem with IL-1β (Hueston and Deak, 2014), however, in contrast to our findings no change was found in IL-1ra expression showing that the effects of stress may be selective for IL-1β and its soluble decoy receptor. In such studies microglia appear to be the major producer of IL-1β as stress-induced central increases in IL-1β can be blocked by pre-treatment with the microglial inhibitor minocycline (Blandino et al., 2006, 2009) while such stressors decrease astrocytic activation as indicated by a reduction in GFAP expression (Blandino et al., 2009). Such findings are in contrast to the results we have presented both here and previously in relation to stimulation of β_2-adrenoceptors with clenbuterol which activates astrocytes and has other anti-inflammatory and neuroprotective actions (Gleeson et al., 2010; McNamee et al., 2010b; Ryan et al., 2013). Moreover, in a study by Hueston and Deak (2014) treatment with dexamethasone had no effect on the stress-induced increase in central IL-1β which is in direct contrast to our results here which showed that clenbuterol-induced IL-1β was blocked by dexamethasone suggesting different mechanisms are at play. However, it is worth noting that previous studies have shown that stress-induced increases in central IL-1β can sensitize the central IL-1 system and the hypothalamic-pituitary-adrenal-axis to subsequent immune challenge resulting in a more exaggerated central response (Johnson et al., 2004). Thus, further studies are necessary to explore the possibility that clenbuterol-induced IL-1β could have a priming effect on the CNS in response to a neuroinflammatory stimulus.

Overall the peripheral effects of clenbuterol (0.5 mg/kg) can be dissociated from its central effects and the increased expression of the anti-inflammatory molecules IL-1ra and IL-1RII may serve to limit the effects of clenbuterol-induced IL-1β on brain function and behaviour. Data following repeated administration of a low dose of clenbuterol (0.03 mg/kg) suggests that it or other selective β_2-adrenoceptor agonists could have the potential to combat the neurodegenerative process without inducing unwanted symptoms of depression and anxiety. However, while both clenbuterol and noradrenaline reuptake inhibitors have neuroprotective (Culmsee et al., 1999a; Gleeson et al., 2010; Zhu et al., 1999) and memory enhancing properties (Clinton et al., 2006; Ramos et al., 2008; Seu et al., 2009), any role that IL-1 system components may have in such neuroprotective or memory enhancing actions of these drugs remains to be determined.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by a grant from the Health Research Board of Ireland awarded to T.J.C.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.02.023.

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