

Noradrenaline reuptake inhibitors limit neuroinflammation in rat cortex following a systemic inflammatory challenge: implications for depression and neurodegeneration

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Abstract

Evidence suggests that noradrenaline has a tonic anti-inflammatory action in the central nervous system (CNS) via its ability to suppress microglial and astrocytic activation, and inhibit production of inflammatory mediators. Consequently it is suggested that noradrenaline may play an endogenous neuroprotective role in CNS disorders where inflammatory events contribute to pathology. Here we demonstrate that acute treatment of rats with the noradrenaline reuptake inhibitors (NRIs) desipramine and atomoxetine elicited anti-inflammatory actions in rat cortex following a systemic challenge with bacterial lipopolysaccharide (LPS). This was characterized by a reduction in cortical gene expression of the pro-inflammatory cytokines interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α), the enzyme inducible nitric oxide synthase (iNOS), and the microglial activation markers CD11b and CD40. These anti-inflammatory actions of NRIs were associated with reduced activation of nuclear factor-kappa B (NF- κ B); a transcription factor that is considered the major regulator of inflammation in the CNS. To determine whether NRI administration directly altered glial expression of these inflammatory markers, primary cortical glial cells were exposed *in vitro* to the NRIs desipramine or atomoxetine. *In vitro* treatment with NRIs largely failed to alter mRNA expression of IL-1 β , TNF- α , iNOS, CD11b and CD40, following stimulation with LPS. Similarly, LPS-induced TNF- α and IL-1 β protein production from glial cells was unaffected by NRI treatment. In contrast, *in vitro* exposure of cultured glial cells to noradrenaline suppressed IL-1 β , TNF- α , iNOS and CD40 expression. These results suggest that *in vivo* administration of NRIs limit inflammatory events in the brain, probably by increasing noradrenaline availability. Overall, this study has yielded significant insights into the ability of noradrenaline-augmentation strategies to limit neuroinflammation.

Received 7 August 2008; Reviewed 25 August 2008; Revised 11 October 2008; Accepted 17 October 2008;
First published online 2 December 2008

Key words: Atomoxetine, cortex, desipramine, inflammation, noradrenaline.

Introduction

There is compelling evidence to suggest that neuroinflammation, characterized by inappropriate microglial activation and inflammatory mediator production,

contributes to the pathophysiology of chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, and also contributes to age-related neurodegeneration (Block *et al.* 2007; Godbout & Johnson, 2006; Griffin & Mrak, 2002). For instance, in Alzheimer's disease, activated microglia and inflammatory cytokines are associated with neuritic plaques, and increased inflammatory cytokine concentrations have also been observed in the cerebrospinal fluid (CSF) of Alzheimer's patients (Heneka &

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O'Banion, 2007). Moreover, epidemiological evidence indicates that there is a lower incidence of Alzheimer's disease in individuals being treated with anti-inflammatory agents to combat peripheral inflammatory diseases such as arthritis (Heneka & O'Banion, 2007). In addition to the role of inflammation in neurodegeneration, a body of evidence indicates that depressive illness is associated with low-grade inflammation that may contribute to depressive symptomatology (Connor & Leonard, 1998; Müller & Schwarz, 2007; Raison *et al.* 2006; Schiepers *et al.* 2005). Consequently, pharmacological strategies that inhibit glial cell activation and production of neurotoxic inflammatory mediators represent an attractive therapeutic target in combating neurodegeneration, and also depressive illness (Block *et al.* 2007; Heneka & O'Banion, 2007; Müller & Schwarz, 2007).

A prevailing theory in the field of neuroinflammation is that certain neurotransmitters elicit anti-inflammatory effects via receptors located on microglia and astrocytes, and thereby play a key role in controlling inflammatory events in the central nervous system (CNS; Feinstein *et al.* 2002; Galea *et al.* 2003). In this regard it is well established that the monoamine neurotransmitter noradrenaline has potent anti-inflammatory properties, and plays an important role in maintaining the immunosuppressive environment within the brain (Feinstein *et al.* 2002; Marien *et al.* 2004). The majority of noradrenaline cell bodies are located in the locus coeruleus (LC) and their axons innervate almost all parts of the brain. Importantly, following its release, noradrenaline is not confined to the vicinity of the synaptic cleft and can reach proximal glial cells (Aoki, 1992). Moreover, the fact that adrenoceptors are expressed on glial cells indicates that their function is subject to modulation by noradrenaline (Mori *et al.* 2002). Specifically, noradrenaline suppresses pro-inflammatory cytokine production from microglia *in vitro* (Dello Russo *et al.* 2004; Mori *et al.* 2002), and can protect cultured neurons from microglial-induced cell death (Madrigal *et al.* 2005; McNamee & Connor, 2008). Moreover, a number of *in vivo* studies also indicate a role for endogenous noradrenaline in maintaining an anti-inflammatory environment in the CNS. For instance, depletion of noradrenaline with *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine (DSP-4) has been shown to augment the inflammatory response to β -amyloid₍₁₋₄₂₎ in rat cortex (Feinstein *et al.* 2002) and it has been shown that noradrenaline depletion promotes an Alzheimer-type pathogenesis in amyloid precursor protein (APP) 23 transgenic mice (Kalinin *et al.* 2007).

Despite the established anti-inflammatory actions of noradrenaline on microglial activation *in vitro* (Dello Russo *et al.* 2004; Mori *et al.* 2002), and the role of endogenous noradrenaline in limiting the CNS inflammation response *in vivo* (Feinstein *et al.* 2002; Kalinin *et al.* 2007), the anti-inflammatory potential of pharmacological agents that enhance central noradrenergic tone have not been examined to date. In this regard, we suggest that pharmacological interventions that increase noradrenaline availability at CNS glia could prove effective in reducing neuroinflammation. In the present investigation we assessed the ability of two structurally dissimilar noradrenaline reuptake inhibitors (NRIs; atomoxetine and desipramine) to alleviate neuroinflammation in rat cortex following a systemic inflammatory challenge with bacterial lipopolysaccharide (LPS). These drugs increase noradrenaline availability by blocking the reuptake of noradrenaline, and are used clinically for the treatment of attention deficit hyperactivity disorder and depression, respectively (Katz *et al.* 2004; Kelsey *et al.* 2004). Administration of desipramine or atomoxetine has been demonstrated to increase extracellular noradrenaline concentration in the cortex, which receives rich noradrenergic innervation from the LC (Kuhar & Lambert, 1999). This brain region is involved in higher cognitive processing that is adversely affected in many neurodegenerative diseases (Burton *et al.* 2004; Whitwell *et al.* 2007).

It is well established that systemic administration of an inflammatory stimulus induces inflammatory cytokine production within the CNS (Breder *et al.* 1994; Hansen *et al.* 1998; Konsman *et al.* 1999). Here we examined gene expression of the pro-inflammatory cytokines TNF- α and IL-1 β and the inducible form of nitric oxide synthase (iNOS); inflammatory mediators implicated in progressing pathology in a number of CNS disease states (Allan *et al.* 2005; Lucas *et al.* 2006; Pannu & Singh, 2006). Activation of the intracellular signalling molecule nuclear factor-kappa B (NF- κ B) that occurs downstream of both LPS binding and cytokine signalling was also assessed. Gene expression of cell surface molecules CD11b and CD40 was measured as these molecules are considered to be phenotypic markers of microglial activation. Moreover, CD40 functions as a co-stimulatory molecule necessary for successful antigen presentation to T cells (Chen *et al.* 2006), and its activation by the CD40 ligand (CD40L) can drive pro-inflammatory cytokine production, whereas CD11b is a complement receptor that is up-regulated on phagocytic microglia (Rotsenker, 2003). In addition, in order to determine if the ability of NRIs to limit neuroinflammation was due to a direct

anti-inflammatory action of the drugs on glial cells or occurs via a noradrenergic mechanism we compared the ability of NRIs and noradrenaline to limit LPS-induced neuroinflammation in a primary glial cell culture system.

Materials and methods

Animals

Male rats postnatal day 1 (Bioresources Unit, Trinity College, Dublin, Ireland) were used to obtain mixed glial cultures. Male Sprague–Dawley rats (Harlan, UK) were used for all *in vivo* experiments. Rats were maintained on a 12 h light–dark cycle (lights on 08:00 hours) in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) and food and water were available *ad libitum* at all times. The experimental protocols were in compliance with the European Communities Council Directive (86/609/EEC).

Treatments

LPS from *Escherichia coli* serotype O111:B4, noradrenaline bitartrate salt hydrate (99%), and desipramine hydrochloride were obtained from Sigma-Aldrich (Ireland). Atomoxetine was donated by Eli Lilly (UK). For *in vivo* experiments desipramine and atomoxetine were dissolved in distilled H_2O (dH_2O), LPS was dissolved in 0.89% NaCl and 0.89% NaCl was administered to control animals. All treatments were administered via the intraperitoneal (i.p.) route in an injection volume of 1 ml/kg. For *in vitro* experiments desipramine and atomoxetine were initially dissolved in H_2O , and LPS dissolved in 0.89% NaCl. All treatments were then diluted to the appropriate concentrations in Dulbecco's Modified Eagle's Medium (DMEM).

Experimental design

Effect of desipramine and atomoxetine on LPS-induced cortical inflammation in vivo

Male Sprague–Dawley rats (200–300 g) were injected with vehicle (dH_2O), desipramine (7.5 or 15 mg/kg) or atomoxetine (5 or 10 mg/kg) before being immediately challenged with LPS (250 $\mu\text{g/kg}$). A vehicle control group (dH_2O and saline) was included to give an estimate of basal expression of inflammatory genes in the rat brain. Rats were killed 4 h post-treatment, the brain removed and a portion of cortex placed in RNAlater (Ambion, UK) to ensure integrity of RNA for gene expression analysis. Another portion of cortex

was rapidly frozen on dry ice for analysis of NF- κB activation.

The experiments presented in this paper were conducted to test the hypothesis that increasing central noradrenergic tone using two structurally diverse NRIs could attenuate experimentally induced neuroinflammation. As the potential of NRIs to suppress inflammatory measures in the brain has not previously been evaluated two doses of each drug were chosen based on their ability to increase extracellular noradrenaline concentrations in the CNS. Specifically, desipramine (10 mg/kg) has been demonstrated to elicit a 2- to 3.5-fold increase in extracellular noradrenaline in various brain regions including the cortex (Beyer *et al.* 2002; Mateo *et al.* 1998). Similarly, systemic injection of atomoxetine (3 mg/kg) has also been shown to elicit a 2.5- to 3-fold increase in noradrenaline in frontal and prefrontal cortices (Bymaster *et al.* 2002; Swanson *et al.* 2006). Systemic administration of these drugs increases extracellular noradrenaline concentrations within 1 h, an effect that lasts for up to 4 h (Beyer *et al.* 2002; Bymaster *et al.* 2002), the time-frame under investigation in these studies.

Cortical gene expression of a number of inflammatory targets was assessed using quantitative real-time polymerase chain reaction (PCR), as the ability to measure brain cytokine protein production is hampered by the lipid nature of brain tissue. We examined the inflammatory response in cortex as this brain region is involved in higher cognitive processing and is adversely affected in many neurodegenerative diseases (Burton *et al.* 2004; Whitwell *et al.* 2007).

Preparation of cortical glia for culture in vitro

Briefly, cortex was dissected out from neonatal rat pups (postnatal day 1) and the meninges and adherent blood vessels removed. Remaining tissue was cross-chopped using a scalpel and placed in 2 ml pre-warmed DMEM (Invitrogen, UK) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Tissue was triturated and passed through a cell strainer with a 40 μm pore size (Becton Dickinson, UK) to obtain a single-cell suspension. Samples were centrifuged at 2000 rpm for 3 min at 20°C , supernatant discarded and the tissue pellet re-suspended in 2 ml of complete pre-warmed, supplemented DMEM (37°C). This was gently triturated until a homogenous cellular suspension was obtained and 50 μl placed in each well of a 24-well plate, pre-coated with poly-L-lysine (Sigma, UK), cell density was typically $2.5 \times 10^5/\text{ml}$. Cells were placed in a humidified incubator (5% $\text{CO}_2/95\%$ air)

and 2 h later were administered 200 μ l supplemented DMEM. DMEM was changed every 3–4 d and cells were matured for 14 d.

Effect of desipramine and atomoxetine on LPS-induced gene expression of TNF- α , IL-1 β , iNOS, CD40 and CD11b from cultured cortical glia

Cortical glia were obtained as described above and treated with desipramine (1 or 10 μ M), atomoxetine (1 or 10 μ M) or DMEM for 30 min prior to LPS (1 μ g/ml) administration and cells were incubated for a further 6 h. At the end of the treatment period culture supernatants were removed and cells harvested for total RNA using a NucleoSpin[®] total RNA isolation kit (Macherney-Nagel, Germany).

Effect of desipramine and atomoxetine on LPS-induced IL-1 β and TNF- α production from cultured cortical glia

Cortical glia were treated with desipramine (1 or 10 μ M), atomoxetine (1 or 10 μ M) or DMEM for 24 h prior to LPS (1 μ g/ml) administration and cells incubated for a further 24 h. At the end of the treatment period culture supernatants were removed and stored at -85°C until cytokine production was determined by enzyme-linked immunosorbent assay (ELISA).

Effect of noradrenaline on LPS-induced IL-1 β and TNF- α production, iNOS protein and CD40 gene expression from cultured cortical glia

Cortical glia were treated with noradrenaline (10 μ M) or DMEM for 2 h prior to LPS (1 μ g/ml) administration and cells incubated for a further 24 h. At the end of the treatment period culture supernatants were removed and stored at -85°C until cytokine production was determined by ELISA. Cells were harvested for Western blotting using a cell lysis buffer [25 mM Hepes, 5 mM MgCl_2 , 5 mM EDTA, 5 mM DTT and 1% protease inhibitor cocktail (Sigma, Ireland) and 1% phosphatase inhibitor I and II cocktail (Sigma)].

Real-time PCR analysis of TNF- α , IL-1 β , iNOS, CD11b and CD40

RNA extraction

Total RNA was extracted from cortex and glial cells using the NucleoSpin[®] total RNA isolation kit (Macherney-Nagel) according to the manufacturer's instructions. This kit also included a DNase step in order to remove any genomic cDNA contamination

from the samples. RNA integrity was assessed by agarose gel electrophoresis and total RNA concentrations were measured by spectrophotometry and stored at -85°C until required for cDNA synthesis.

cDNA synthesis

Total RNA concentrations were adjusted to a standard concentration prior to cDNA synthesis. 0.5–1 μ g total RNA was then reverse-transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, Germany) according to the manufacturer's instructions. The resultant cDNA was stored at -20°C until required for real-time PCR.

Real-time PCR

Real-time PCR was performed using Taqman Gene expression assays (Applied Biosystems) on an ABI Prism 7300 instrument (Applied Biosystems) as previously described (Boyle & Connor, 2007). The assay IDs for the genes examined were as follows: TNF- α (Rn99999017_m1), IL-1 β (Rn00580432_m1), iNOS (Rn00561646_m1), CD40 (Rn01423583_m1), CD11b (Rn00709342_m1). Gene expression was calculated relative to the endogenous control samples (β -actin) and to the control sample to give a relative quantification (RQ) value (2^{-DDC_T} , where C_T is the threshold cycle).

ELISA measurements of IL-1 β and TNF- α

IL-1 β and TNF- α concentrations were measured using ELISA kits from R & D Systems (UK) and BD Pharmingen (UK), respectively. Assays were performed according to manufacturers' instructions and absorbance read at 450 nm using a microplate reader. Absorbance was then calculated as a concentration (pg/ml) using a standard curve derived using GraphPad Prism software, version 4.00 (GraphPad Software Inc., USA). This was then converted to % LPS control.

ELISA measurement of intracellular/nuclear NF- κ B

Nuclear fractions were prepared from homogenized cortex using an NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, UK) according to the manufacturer's instructions. NF- κ B p65 subunit binding in nuclear fractions was measured using an EZ-Detect NF- κ B p65 Transcription Factor kit (Pierce) according to the manufacturer's instructions. Fluorescence/mg protein was then converted to % LPS control.

Analysis of iNOS by Western immunoblotting

Cells were harvested as described earlier and equalized for total protein content following measurement of protein using the BCA protein assay kit (Pierce). Samples were separated using gel electrophoresis and transferred to nitrocellulose as previously described (Boyle & Connor, 2007). The nitrocellulose membrane was incubated overnight with rabbit monoclonal iNOS antibody [Transduction Laboratories (UK), 1:1000 dilution in Tris-buffered saline (TBS) containing 2% bovine serum albumin (BSA)] and immunoreactive bands detected using a peroxidase-linked anti-rabbit IgG (1:2000 dilution in TBS containing 2% BSA). Nitrocellulose membranes were stripped using ReBlot Plus Strong (Chemicon, USA) and probed with a mouse monoclonal IgG antibody that targets rat β -actin (Sigma, 1:1000 dilution in TBS containing 2% BSA) and immunoreactive bands detected using a peroxidase-linked anti-mouse IgG (1:2000 dilution in TBS containing 2% BSA).

Protein bands were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce). Immunoblots were exposed to film (Amersham Biosciences, UK) and processed using a Fuji X-ray processor. Bands were quantified by densitometric analysis using Labworks version 4.6 (UVP, UK). Labworks provides a single value (in arbitrary units) representing the density of each blot.

Statistical analysis of data

All values are expressed as mean $\pm$ standard error of the mean (S.E.M.) and converted to a % of LPS, i.e. in the absence of drug treatment and presence of LPS. Data were analysed using a one- or two-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) *post-hoc* test, where appropriate. A *p* value <0.05 was considered statistically significant.

Results

Desipramine and atomoxetine suppress LPS-induced inflammatory gene expression and intracellular signalling in vivo

Desipramine and atomoxetine suppress LPS-induced TNF- α , IL-1 β and iNOS gene expression in rat cortex

We observed a robust induction of TNF- α (7-fold), IL-1 β (4-fold) and iNOS (23-fold) in cortical tissue following LPS treatment. Our data demonstrate that administration of desipramine (15 mg/kg) or

atomoxetine (10 mg/kg) reduced LPS-induced TNF- α gene expression (Fig. 1a), and desipramine (7.5 and 15 mg/kg) or atomoxetine (5 and 10 mg/kg) suppressed IL-1 β and iNOS gene expression (Fig. 1b,c).

Desipramine and atomoxetine suppress LPS-induced CD11b and CD40 gene expression

We observed an induction of the microglial activation markers CD11b (2-fold) and CD40 (5-fold) in cortical tissue following LPS treatment. Our data demonstrate that administration of desipramine (7.5 and 15 mg/kg) or atomoxetine (5 and 10 mg/kg) reduced LPS induction of CD11b and CD40 gene expression (Fig. 2a,b).

Desipramine and atomoxetine suppress LPS-induced NF- κ B activation

To characterize the means by which desipramine and atomoxetine might modulate pro-inflammatory cytokines, iNOS and CD40, we examined the ability of these agents to modulate NF- κ B activation. We observed an increase in NF- κ B activation (1.5-fold) in cortical tissue following LPS administration. Our data demonstrate that administration of desipramine (15 mg/kg) or atomoxetine (5 and 10 mg/kg) reduced LPS-induced activation of NF- κ B (Fig. 3).

Noradrenaline, but not desipramine or atomoxetine suppresses LPS-induced inflammatory gene expression and protein production in cultured cortical glia

We used mixed cortical glia in these experiments which typically contain 70% astrocytes and 30% microglia, approximating the astrocyte: microglia ratio *in vivo*.

Desipramine and atomoxetine fail to alter gene expression of pro-inflammatory cytokines, iNOS and microglial activation markers CD40 and CD11b from cortical mixed glia

In order to determine whether desipramine and atomoxetine were acting directly on glial cells in the brain to suppress LPS-induced gene expression of TNF- α , IL-1 β , iNOS and the microglial activation markers CD11b and CD40, we examined the action of these drugs on mixed cortical glia. In contrast to the profound anti-inflammatory action of NRIs on inflammation in the intact CNS, we observed no significant change in gene expression of inflammatory measures following direct NRI administration to cultured cortical glia (Table 1).

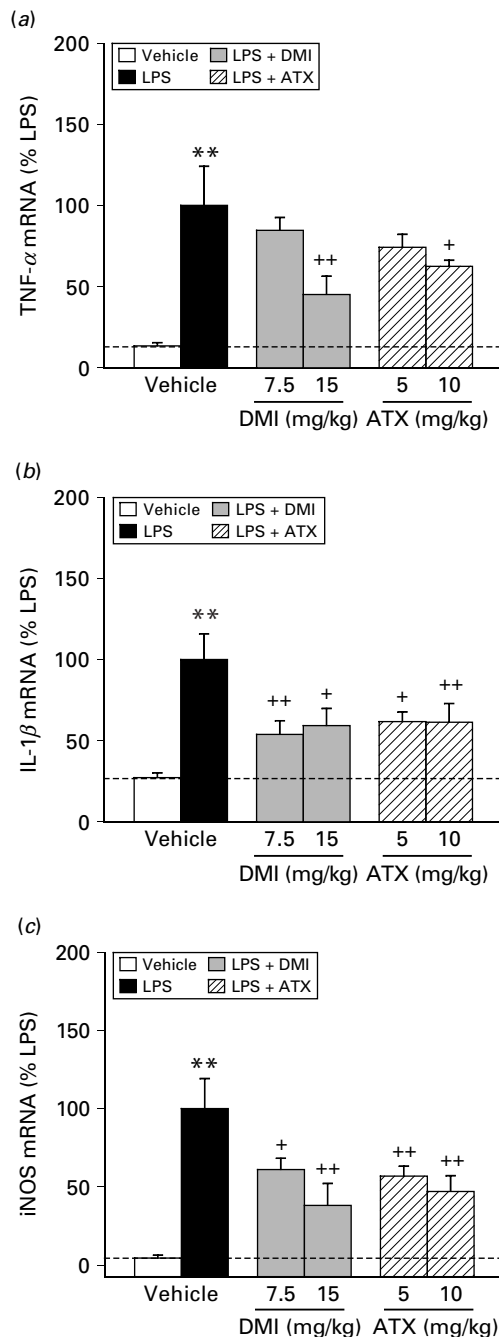


Fig. 1. Desipramine (DMI) and atomoxetine (ATX) attenuated lipopolysaccharide (LPS)-induced TNF- α , IL-1 β and iNOS mRNA expression in rat cortex. Desipramine, atomoxetine or vehicle was administered to rats immediately prior to a challenge with LPS (250 μ g/kg). (a) Desipramine (15 mg/kg) and atomoxetine (10 mg/kg) significantly attenuated LPS-induced TNF- α mRNA expression. (b) Desipramine (7.5 and 15 mg/kg) and atomoxetine (5 and 10 mg/kg) significantly attenuated LPS-induced IL-1 β mRNA expression and (c) desipramine (7.5 and 15 mg/kg) and atomoxetine (5 and 10 mg/kg) significantly attenuated

Desipramine and atomoxetine fail to alter production of the pro-inflammatory cytokines TNF- α and IL-1 β from cortical mixed glia

In order to determine whether desipramine or atomoxetine could alter protein production of TNF- α and IL-1 β from mixed cortical glia, we examined the action of these drugs on mixed glia. We observed no significant change in protein production of either TNF- α or IL-1 β (Table 2).

Noradrenaline suppresses LPS-induced TNF- α and IL-1 β production, iNOS expression and CD40 gene expression from cortical mixed glia

To determine whether *in vitro* exposure of mixed glia to noradrenaline would mimic the effects of desipramine or atomoxetine on intact brain, we examined the effect of *in vitro* exposure of glia to noradrenaline prior to LPS treatment. We observed that pre-treatment of glia with noradrenaline robustly reduced LPS-induced TNF- α and IL-1 β production, iNOS expression and CD40 gene expression (Fig. 4).

Discussion

The major finding of the present study was that *in vivo* administration of the NRIs desipramine and atomoxetine attenuated gene expression of pro-inflammatory cytokines (IL-1 β and TNF- α), iNOS and microglial activation markers (CD40 and CD11b) in rat cortex following a systemic LPS challenge. These findings are of significance as IL-1 β and TNF- α are involved in initiating and maintaining the inflammatory cascade and also have the potential to cause cell death, following TNF- α receptor 1 (Yang *et al.* 2002) or IL-1 β type 1 receptor activation (Thornton *et al.* 2006). Evidence from the clinic and animal models indicates distinct roles for these cytokines in neurodegenerative disorders. For instance, IL-1 β is implicated as a central player in animal models of Alzheimer's disease and multiple sclerosis (Griffin, 2006; Lucas *et al.* 2006; Mulcahy *et al.* 2003) whereas TNF- α is indicated as a major contributor to pathology in Parkinson's disease

LPS-induced iNOS mRNA in rat cortex 4 h post-treatment. One-way ANOVA revealed a significant effect of treatment on (a) TNF- α [$F(5, 28) = 9.4, p < 0.0001$], (b) IL-1 β [$F(5, 28) = 5.6, p < 0.01$] and (c) iNOS [$F(5, 27) = 8.1, p < 0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean ($n = 4-6$). ** $p < 0.01$ vs. vehicle/vehicle; + $p < 0.05$, ++ $p < 0.01$ vs. vehicle/LPS group (Fisher's LSD post-hoc test).

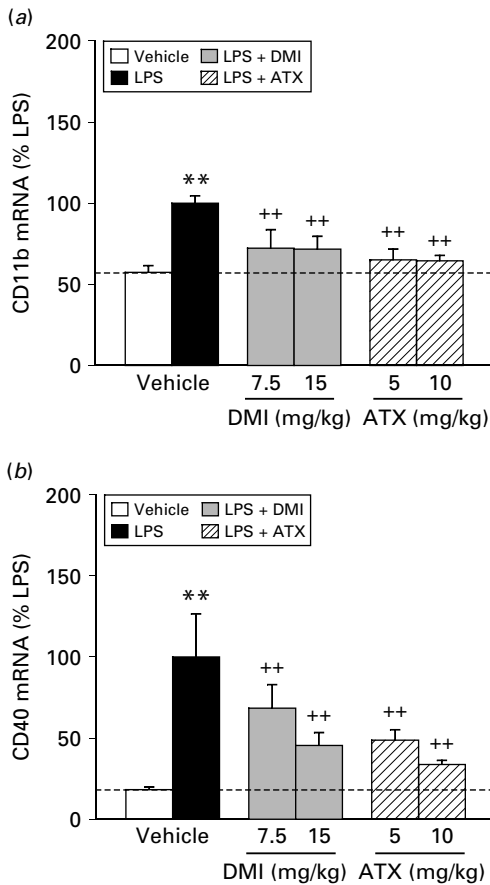


Fig. 2. Desipramine (DMI) and atomoxetine (ATX) attenuated lipopolysaccharide (LPS)-induced CD11b and CD40 mRNA expression in rat cortex. Desipramine, atomoxetine or vehicle was administered to rats immediately prior to a challenge with LPS (250 μ g/kg). (a) Desipramine (7.5 and 5 mg/kg) and atomoxetine (5 and 10 mg/kg) significantly attenuated LPS-induced CD11b mRNA expression. (b) Desipramine (7.5 and 15 mg/kg) and atomoxetine (5 and 10 mg/kg) significantly attenuated LPS-induced CD40 mRNA expression in rat cortex 4 h post-treatment. One-way ANOVA revealed a significant effect of treatment on (a) CD11b [$F(5, 25) = 5.2$, $p < 0.01$], (b) CD40 [$F(5, 28) = 8.7$, $p < 0.0001$] mRNA expression. Data expressed as means (% LPS) and standard error of the mean ($n = 5-6$). ** $p < 0.01$ vs. vehicle/vehicle; ++ $p < 0.01$ vs. vehicle/LPS group (Fisher's LSD *post-hoc* test).

(Hunot & Hirsch, 2003). Similarly, the excessive production of nitric oxide that occurs following iNOS induction can exert a number of cytotoxic effects, and both clinical (Iravani *et al.* 2002; Qureshi *et al.* 1995) and preclinical studies (Dehmer *et al.* 2000) indicate an important role for nitric oxide in mediating neurotoxicity. The data presented here suggest that the ability of desipramine and atomoxetine to attenuate

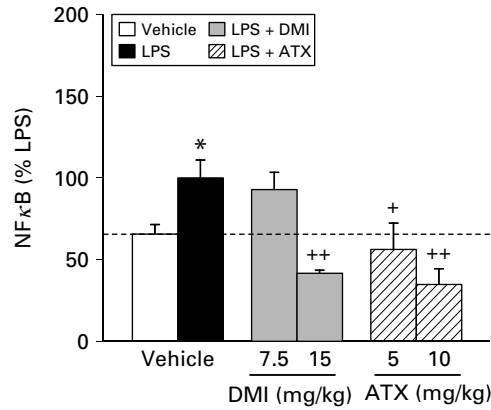


Fig. 3. Desipramine (DMI) and atomoxetine (ATX) attenuated lipopolysaccharide (LPS)-induced NF- κ B activation in rat cortex. Desipramine, atomoxetine or vehicle was administered to rats immediately prior to a challenge with LPS (250 μ g/kg). Desipramine (15 mg/kg) and atomoxetine (5 and 10 mg/kg) significantly attenuated LPS-induced NF- κ B activation as measured by ELISA. One-way ANOVA revealed a significant effect of treatment on NF- κ B activation [$F(5, 28) = 6.57$, $p < 0.001$]. Data expressed as means (% LPS) and standard error of the mean ($n = 4-6$). * $p < 0.05$ vs. vehicle/vehicle; + $p < 0.05$, ++ $p < 0.01$ vs. vehicle/LPS group (Fisher's LSD *post-hoc* test).

pro-inflammatory cytokine and iNOS gene expression may have neuroprotective actions following an inflammatory insult.

We propose that desipramine and atomoxetine exert their anti-inflammatory actions by increasing noradrenaline availability to glial cells as direct administration of these drugs did not alter any of the inflammatory indices assessed in cultured mixed cortical glia. These findings are consistent with previous work indicating that *in vitro* exposure to desipramine or the more selective NRI reboxetine failed to alter IL-1 β or TNF- α production from LPS-stimulated diluted human blood (Diamond *et al.* 2006). Moreover, the fact that atomoxetine elicited qualitatively similar anti-inflammatory actions to desipramine argues against the possibility that non-noradrenergic actions of desipramine such as its antagonistic action at histamine, muscarinic or adrenergic receptors (see Leonard, 2003) contributed to its anti-inflammatory actions. This said, some recent studies question the specificity of atomoxetine as an NRI, and demonstrate that atomoxetine also increases histamine and dopamine concentrations in rat brain (Horner *et al.* 2007; Liu *et al.* 2008; Swanson *et al.* 2006). Whilst it is unlikely that dopamine or histamine play a significant role in the anti-inflammatory actions of atomoxetine in the CNS,

Table 1. Desipramine (DMI) and atomoxetine (ATX) failed to alter gene expression of pro-inflammatory cytokines, iNOS and the microglial activation markers CD11b and CD40 in cortical mixed glia

Target (% LPS)	Vehicle	DMI (1 μ M)	DMI (10 μ M)	ATX (1 μ M)	ATX (10 μ M)	ANOVA
TNF- α	100 $\pm$ 9	108 $\pm$ 21	103 $\pm$ 12	110 $\pm$ 12	111 $\pm$ 15	$F(4, 23) = 0.25, p = 0.9$
IL-1 β	100 $\pm$ 17	81 $\pm$ 12	100 $\pm$ 19	103 $\pm$ 22	101 $\pm$ 11	$F(4, 23) = 0.28, p = 0.9$
iNOS	100 $\pm$ 25	65 $\pm$ 17	99 $\pm$ 26	86 $\pm$ 28	84 $\pm$ 14	$F(4, 23) = 0.36, p = 0.8$
CD11b	100 $\pm$ 22	74 $\pm$ 13	97 $\pm$ 19	97 $\pm$ 26	79 $\pm$ 10	$F(4, 23) = 0.38, p = 0.8$
CD40	100 $\pm$ 18	83 $\pm$ 7	105 $\pm$ 19	90 $\pm$ 19	83 $\pm$ 28	$F(4, 23) = 0.3, p = 0.9$

Glial cells were incubated with desipramine (1 or 10 μ M), atomoxetine (1 or 10 μ M) or vehicle 30 min prior to stimulation with lipopolysaccharide (LPS) (1 μ g/ml), and cells incubated with these treatments for 6 h. Treatment with desipramine or atomoxetine did not alter LPS-induced inflammatory gene expression from cortical mixed glia. Data expressed as mean (% LPS control) with standard error of the mean ($n = 6$). One-way ANOVA revealed no significant effect of treatments on target gene expression.

Table 2. Desipramine (DMI) and atomoxetine (ATX) failed to alter production of the pro-inflammatory cytokines TNF- α and IL-1 β from cortical mixed glia

Cytokine (% LPS)	Vehicle	DMI (1 μ M)	DMI (10 μ M)	ATX (1 μ M)	ATX (10 μ M)	ANOVA
TNF- α	100 $\pm$ 16	151 $\pm$ 18	148 $\pm$ 26	126 $\pm$ 10	137 $\pm$ 15	$F(4, 25) = 1.37, p = 0.27$
IL-1 β	100 $\pm$ 16	97 $\pm$ 13	68 $\pm$ 14	79 $\pm$ 11	108 $\pm$ 9	$F(4, 25) = 1.62, p = 0.2$

Glial cells were incubated with desipramine (1 or 10 μ M), atomoxetine (1 or 10 μ M) or vehicle 24 h prior to stimulation with lipopolysaccharide (LPS) (1 μ g/ml), and cells incubated with these treatments for a further 24 h. Treatment with desipramine or atomoxetine did not alter LPS-induced TNF- α or IL-1 β protein production from cortical mixed glia. Data expressed as mean (% LPS control) with standard error of the mean ($n = 6$). One-way ANOVA revealed no significant effect of treatments on cytokine protein production. Vehicle/LPS-treated group: TNF- α concentration was 925 ± 147 pg/ml and IL-1 β concentration was 308 ± 48 pg/ml.

we can not completely rule out this possibility at this stage.

Although increases in extracellular noradrenaline concentrations persist for 4 h following NRI administration (Beyer *et al.* 2002; Bymaster *et al.* 2002), it is possible that the anti-inflammatory effects of such treatment may persist at later time-points by dampening the early inflammatory phase, thereby preventing the spread of inflammation throughout the brain parenchyma. It has been previously demonstrated that systemic administration of LPS causes an acute inflammatory response in endothelial cells of the blood-brain barrier and also within circumventricular organs, before affecting parenchymal cells (Nadeau &

Rivest, 1999; Quan *et al.* 1998). Additional studies are necessary to dissect the ability of acute NRI treatment to alter temporal aspects of inflammatory response in the CNS, such as induction of the LPS receptor CD14 (Nadeau & Rivest, 2002).

In contrast to the failure of NRIs to alter the inflammatory response from glia *in vitro*, direct administration of noradrenaline to these cells suppressed IL-1 β and TNF- α production, iNOS expression, and gene expression of the microglial activation marker CD40. This finding concurs with a body of literature demonstrating reduced expression of TNF- α , IL-1 β , iNOS and nitric oxide from microglia (Dello Russo *et al.* 2004; Mori *et al.* 2002) and astrocytes (Akama

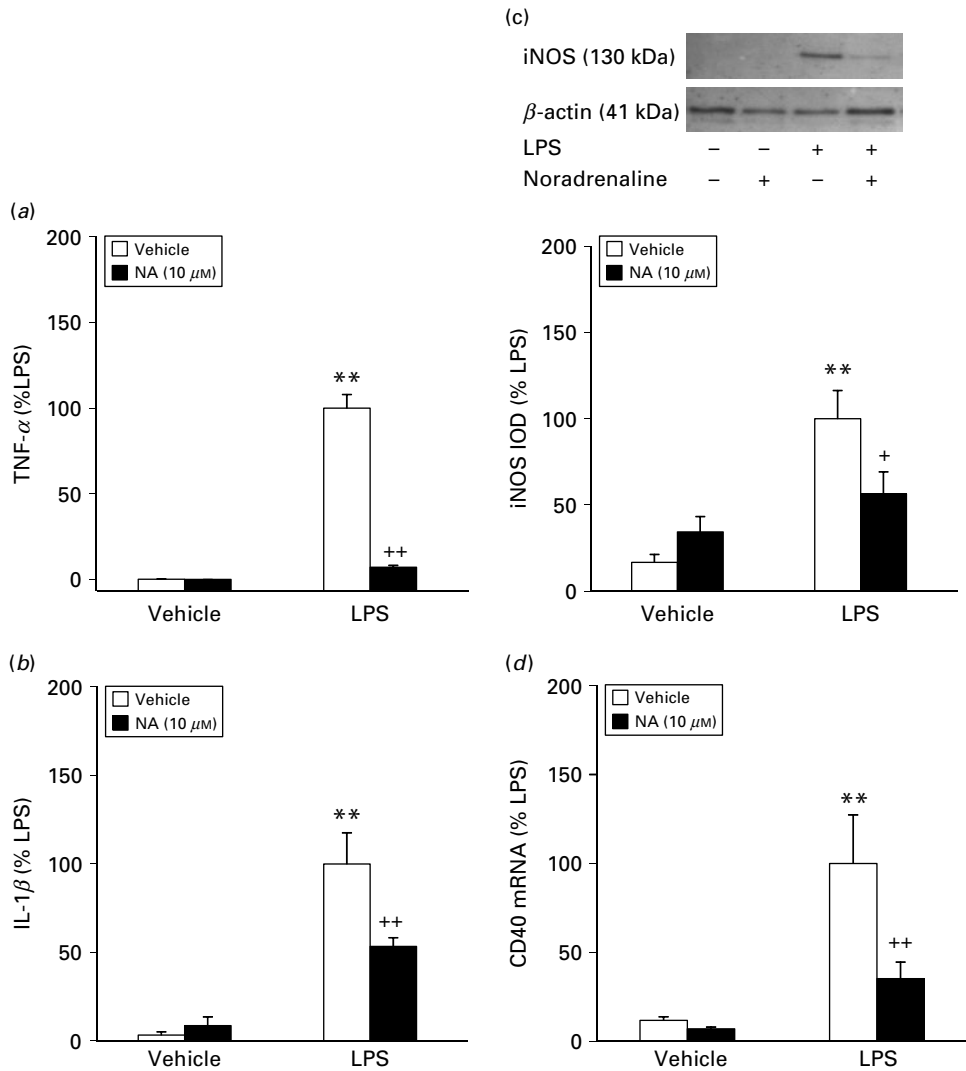


Fig. 4. Noradrenaline (NA) suppresses TNF- α and IL-1 β production, iNOS expression and CD40 gene expression in mixed cortical glial cells. Vehicle or noradrenaline (10 μ M) were administered either 30 min (mRNA measurements) or 2 h (protein) prior to vehicle or lipopolysaccharide (LPS) (1 μ g/ml) and cells were incubated with these treatments for a further 6 h or 24 h, for mRNA or protein measurements, respectively. Noradrenaline pre-treatment significantly attenuated (a) TNF- α protein production, (b) IL-1 β protein production, (c) iNOS protein expression and (d) CD40 gene expression from cortical mixed glia. Data expressed as mean (% LPS control) with standard error of the mean ($n=4-6$). Two-way ANOVA revealed a significant noradrenaline $\times$ LPS interaction on (a) TNF- α production [$F(1, 28)=133.66$, $p<0.0001$], (b) IL-1 β production [$F(1, 28)=7.47$, $p<0.05$], (c) iNOS expression [$F(1, 25)=6.27$, $p<0.05$] and (d) CD40 expression [$F(1, 20)=4.31$, $p=0.05$]. Data expressed as means (% LPS) and standard error of the mean ($n=6-8$). ** $p<0.01$ vs. vehicle/vehicle; + $p<0.05$, ++ $p<0.01$ vs. vehicle/LPS group (Fisher's LSD *post-hoc* test).

& Van Eldik, 2000; Feinstein *et al.* 1993; Nakamura *et al.* 1998) following treatment with noradrenaline or β -adrenoceptor agonists. Although the ability of noradrenaline or noradrenaline-enhancing agents to alter CD40 or CD11b expression *in vivo* or *in vitro* has not previously been reported, we suggest that *in vivo* suppression of CD40 and CD11b gene expression following desipramine and atomoxetine treatment

indicates an attenuated microglial response to LPS treatment. The ability of NRIs to reduce microglial activation is of significance, considering evidence that inhibition of microglial activation represents a therapeutic strategy to combat neurodegeneration (Liu & Hong, 2003). As *in vitro* administration of NRIs does not alter these markers of microglial activation we postulate that the suppression observed *in vivo* results

from extracellular noradrenaline exerting a quiescent action on glia. This is supported by the observation that *in vitro* administration of noradrenaline suppresses gene expression of CD40 on cultured glia.

Although no previous studies have examined the potential of noradrenaline-enhancing strategies to attenuate indices of neuroinflammation *in vivo*, our results correspond favourably with those of Heneka *et al.* (2002) who demonstrated a role for endogenous noradrenaline in cortical inflammation. By depleting endogenous noradrenaline using DSP-4 Heneka and colleagues demonstrated a role for endogenous noradrenaline in modulating IL-1 β , IL-6 and iNOS gene expression and protein following a cortical injection of β -amyloid peptide. Furthermore, the authors of that study reported that co-injection of either noradrenaline or a β -adrenoceptor agonist ameliorated the heightened level of inflammation observed in these DSP-4-lesioned animals (Heneka *et al.* 2002). Moreover, mice expressing human amyloid precursor protein (hAPP) that were continuously depleted of noradrenaline by chronic DSP-4 administration, had five times more β -amyloid deposition than noradrenaline-intact hAPP-expressing mice (Kalinin *et al.* 2007). Here we utilized a novel method to enhance endogenous noradrenaline within the CNS, and show attenuated inflammatory response to peripheral LPS following NRI treatment. We postulate that an increase in extracellular noradrenaline following NRI treatment acts on glial cells to suppress inflammation in the brain, which, in agreement with the literature, suggests an anti-inflammatory role for noradrenaline in the CNS (Feinstein *et al.* 2002; Marien *et al.* 2004).

It is well established that the intracellular signalling molecule NF- κ B is activated following LPS and IL-1 β signalling (Huang *et al.* 2004; Palsson-McDermott & O'Neill, 2004). The data presented here suggests that NRI administration modulates NF- κ B activation following LPS treatment *in vivo*. Indeed, previous investigators have proposed NF- κ B as a target for noradrenergic modulation following an inflammatory stimulus in astrocytes (Feinstein *et al.* 2002), T lymphocytes (Neumann *et al.* 1995) and human promonocytic THP-1 cells (Farmer & Pugin, 2000). The attenuation of TNF- α expression by NRI treatment was slightly less than that of IL-1 β or iNOS in the present study. Similarly, the low dose of desipramine was effective in attenuating expression of pro-inflammatory cytokines and microglial activation markers, but not NF- κ B. The ability of NRIs to exert quantitatively different effects on expression of these inflammatory markers could result from either the ability of LPS and IL-1 β receptor activation to induce

mitogen-activated protein kinases (MAPKs) and interferon regulatory factor 3 in addition to NF- κ B intracellular signalling (Liew *et al.* 2005; Moynagh, 2005). Further investigations are merited to investigate the possibility that noradrenaline may alter these signalling pathways. Temporal differences in NF- κ B activation and gene expression of pro-inflammatory gene expression and microglial markers may also account for the quantitative differences observed in these markers following NRI treatment.

In conclusion, the results of the present investigation clearly demonstrate that noradrenaline *in vitro*, and noradrenaline-enhancing agents *in vivo*, inhibit inflammatory processes in glial cells and intact brain respectively. Considering the evidence that microglial activation and production of pro-inflammatory cytokines and nitric oxide contribute to pathology in a number of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, it is suggested that the ability of two different classes of NRI to inhibit IL-1 β , TNF- α , iNOS and microglial activation in the CNS could be of therapeutic benefit in combating inflammation-related neurodegeneration and depression. In this regard, desipramine inhibits LPS-induced apoptosis in hippocampal-derived neural stem cells (Huang *et al.* 2007), and nortryptiline, a NRI related to desipramine, has neuroprotective efficacy in a mouse model of amyotrophic lateral sclerosis (Wang *et al.* 2007). In addition, we have previously demonstrated that desipramine treatment completely blockades LPS-induced depressive-like behaviour in rats, and may therefore protect against inflammation-induced depression (Shen *et al.* 1999).

Acknowledgements

The authors thank Science Foundation Ireland for financial support and Eli Lilly (USA) for the gift of atomoxetine.

Statement of Interest

None.

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