

The involvement of norepinephrine and microglia in hypothalamic and splenic IL-1 β responses to stress

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Abstract

The noradrenergic system plays an integral role in the stress response and modulates expression of proinflammatory cytokines. Recent work from our laboratory and others has shown that certain stressors increase the expression of the proinflammatory cytokine interleukin-1 β (IL-1 β) in the hypothalamus and spleen. One goal of the following studies was to assess the role of norepinephrine in stress-elicited increases in IL-1 β . To do this, adult male Sprague–Dawley rats were injected with propranolol (20 mg/kg i.p.) or desipramine (20 mg/kg s.c.) and exposed to 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each). We found that propranolol blocked the IL-1 β response to footshock in both the hypothalamus and the spleen, while the noradrenergic reuptake inhibitor desipramine significantly augmented the footshock-induced IL-1 β response in both of these sites. Our second goal was to determine whether these effects would also be blocked by administration of a putative microglial inhibitor (minocycline). Minocycline (40 mg/kg i.p.) completely reversed the footshock-induced increase in hypothalamic IL-1 β but had no effect on the IL-1 β response in the spleen. Moreover, lack of an effect of minocycline on conditioned fear responding suggests that the effect of this drug cannot be explained by nonspecific sedative properties produced by the drug. Together, these data suggest that NE powerfully modulates the hypothalamic and splenic IL-1 β response to stress, and that microglia may be a primary cellular source of central IL-1 β in response to footshock.

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1. Introduction

During times of stress, the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS) coordinate behavioral and physiological responses of the organism in order to facilitate survival from an impending threat. Recent evidence suggests that immune-related factors such as pro-inflammatory cytokines are also increased by stress, even though no apparent infection has been incurred. For instance, increased pro-inflammatory cytokines have been observed in both blood (Morrow et al., 1993) and brain (Deak et al., 2003; Deak et al., 2005b; Nguyen et al., 1998; Nguyen et al., 2000; O'Connor et al.,

2003; Shintani et al., 1995) after stressor exposure. Other signs of immune activation have also been observed following stressor exposure, including leukocytosis (Maier and Watkins, 1998), febrile responses (Deak et al., 1997; Oka et al., 2001) and increased expression of several immune-related transcripts (Reyes et al., 2003). At one level or another, these tell-tale signs of immune activation following stressor exposure are thought to be involved in the facilitation of immune function produced by these very same stressors (Deak et al., 1999b; Dhabhar et al., 1995; Dhabhar et al., 1996; Dhabhar and McEwen, 1997; Johnson et al., 2004).

Increased expression of interleukin-1 β (IL-1 β) appears to play a particularly prominent role in coordinating centrally mediated responses to stress. For instance, increased IL-1 β has been observed in the hypothalamus after a variety of stressors such as tailshock (Nguyen et al., 1998; Nguyen et

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al., 2000; O'Connor et al., 2003), footshock (Deak et al., 2003), and immobilization (Shintani et al., 1995) while other stressors such as maternal separation (Hennessy et al., 2004), predator exposure (Plata-Salaman et al., 2000), forced swim (Deak et al., 2003) and simple restraint (Deak et al., 2005b) had no effect on central IL-1 expression. Functional studies have shown that IL-1 receptor antagonist (IL-1ra) blunted the hypothalamic monoamine and adrenocorticotrophic hormone (ACTH) response to immobilization stress (Shintani et al., 1995), and reversed behavioral signs of learned helplessness incurred by inescapable tailshock exposure (Maier and Watkins, 1995). Furthermore, Johnson et al. (2004) have shown that central IL-1ra blocked tailshock-induced sensitization of pituitary–adrenal and cytokine responses to later lipopolysaccharide (LPS) challenge. Using a more broad inhibitor of central inflammatory processes, Milligan et al. (1998) showed that central administration of alpha-melanocyte stimulating hormone (α -MSH) blocked adipisia, aphagia and fever responses produced by inescapable tailshock. We have observed similar effects in the guinea pig where α -MSH reversed sickness-like behavioral responses observed during maternal separation (Schiml-Webb et al., *in press*). These effects of α -MSH in reversing stress-related behavioral changes are thought to reflect anti-inflammatory properties of α -MSH through physiological antagonism of IL-1 β action (Catania et al., 1999; Macaluso et al., 1994). Finally, at least some behavioral consequences of stressor exposure can be mimicked by central injection of IL-1 β , including learning deficits comparable to those observed after social isolation (Pugh et al., 1999) and learned helplessness-like deficits (Bonaccorso et al., 2003). Together, these findings provide converging lines of evidence that IL-1 β is an important mediator of behavioral and physiological responses to stress. It is therefore of great interest to explore the mechanisms by which stressors regulate the production of IL-1 β in the CNS and periphery.

Catecholamines powerfully regulate immune function and a variety of recent studies have implicated adrenergic receptor activation as a critical step in the regulation of cytokines. For instance, peripheral pretreatment with an α 1-adrenergic receptor antagonist blocked the plasma IL-1 and IL-6 response to tailshock, but had no effect on the IL-1 response in several CNS structures of the same animals (Johnson et al., *in press*). Conversely, these same authors showed that peripheral pretreatment with a β -adrenergic receptor antagonist blocked the cytokine response in several structures of the CNS, while the plasma cytokine response to tailshock was unaffected by this drug. In the periphery, activation of β -adrenergic receptors in the spleen has been shown to elicit a robust increase in many pro-inflammatory cytokines [reviewed by (Sanders and Straub, 2002)], and sympathetic ablation appears to reverse increased IL-1 expression in the spleen produced by oscillation stress (Jung et al., 2000). In the CNS, *i.c.v.* (intracerebral ventricular) injection of the β -adrenergic agonist isoproter-

enol increased IL-1 β mRNA expression with some evidence implicating microglia as the likely cellular source of IL-1 β (Maruta et al., 1997). Together, these data suggest that catecholamines are critically involved in cytokine responses to stress, but that the adrenergic receptor subtype responsible for cytokine induction may depend on the site being examined. In light of these findings, it is rather surprising that the role of microglia in stress-induced cytokine production has not been explored previously.

We have previously shown that exposure to footshock increased hypothalamic and splenic IL-1 β concentrations in the Sprague–Dawley rat, while IL-1 β concentrations in other regions of the CNS (cortex and hippocampus) and peripheral organs (adrenal and pituitary glands) were unaffected (Deak et al., 2003). The first goal of the following series of experiments, therefore, was to further clarify the involvement of catecholamines in hypothalamic and splenic IL-1 β responses to footshock. Specifically, we hypothesized that the hypothalamic and splenic IL-1 β response to footshock would be (i) blocked by pretreatment with propranolol, a selective β -adrenergic receptor antagonist, and (ii) augmented by pretreatment with desipramine, a tricyclic antidepressant with noradrenergic reuptake inhibition as its primary mechanism of action. Since there is some evidence to suggest that the β -adrenergic mediated increase in hypothalamic IL-1 β may occur as a result of direct action on microglia (Maruta et al., 1997), we further hypothesized that (iii) systemic administration of a putative microglial inhibitor (minocycline) would block hypothalamic, but not splenic, IL-1 β responses to footshock. Because we sought to block cytokine changes in both the CNS and periphery and each of these agents have excellent penetration of the CNS when administered peripherally, all drugs were administered intraperitoneal (*i.p.*) or subcutaneous at the nape of the neck (*s.c.*). Plasma concentrations of corticosterone (CORT) and glucose were also evaluated as general indicators of the stress response. Since our previous work indicated that footshock failed to increase circulating IL-1 (Deak et al., 2003), plasma cytokines were not examined.

2. Materials and methods

2.1. Subjects

Adult male Sprague–Dawley rats (300–450 g) were born and bred in the animal facility in the Department of Psychology at SUNY-Binghamton using breeders purchased from Harlan. Colony conditions were maintained at 22 ± 1 °C with a 14:10 light–dark cycle (lights on 06:00–20:00 h) for optimal breeding (Sharp and LaRegina, 1998). Animals were pair housed in standard transparent Plexiglas cages and had access to food and water *ad libitum*. In all experiments, rats were handled briefly for 3–5 min on each of 2 days prior to experimentation and stress to the animals was minimized whenever possible. All experimental procedures

were approved by the Institutional Animal Care and Use Committee at Binghamton University.

2.2. Footshock paradigm

Rats were exposed to 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h. The footshock chambers were standard operant chambers measuring 23.5 cm × 20.5 cm × 18 cm (*L* × *W* × *H*; Ralph Gerbrands, Model C, Arlington, MA, USA) that were adapted to deliver shock through the grid floor (18 bars spaced 1 cm apart on center, with a diameter of 1.0 mm each). All current was delivered to the grid floor by a shocker (BRS/LVE, Model SGS-004, Beltsville, MD, USA) driven by a personal computer interface.

2.3. Tissue collection and measurement of IL-1 β

Rats were sacrificed by rapid decapitation (unanaesthetized) immediately after footshock exposure and trunk blood was collected into EDTA coated Vacutainers and stored on ice. Plasma was immediately separated through refrigerated centrifugation, aliquoted and stored at -20°C until time of assay. Brain and peripheral tissue were quickly collected and dissected on a cold plate and flash frozen. All tissue was stored at -70°C until the time of assay. On the day of the assay, tissue was thawed in 0.25–1.0 ml of ice cold buffer (pH 7.2, 4°C) containing 50 nM Tris, 1 mM EDTA, 6 mM MgCl_2 , 1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ antipain, 1 $\mu\text{g/ml}$ aprotinin and 1 mg/ml of soybean trypsin inhibitor. The tissue was then sonicated for 5–10 s using an ultrasonic dismembrator (Fisher, Model 100) at a setting of 5. Sonicated samples were centrifuged for 10 min at 14,000 rpm (4°C) and supernatants extracted. IL-1 β was measured using a commercially available rat IL-1 β enzyme-linked immunosorbent assay (ELISA) kit (R and D Systems, Minneapolis, MN, USA) and absorbance was read at 450 nm. Total protein content from each sample was measured using the method of Bradford (Bradford, 1976). All data are expressed as picogram of IL-1/100 μg of total protein. This procedure was previously validated as an effective method for the detection of IL-1 in both central and peripheral tissue compartments (Deak et al., 2003; Hosoi et al., 2000; Nguyen et al., 1998; Nguyen et al., 2000; Pugh et al., 1999). The inter- and intra-assay coefficients of variation were both less than 9% and the detection limits of the assay are 5 pg/ml according to manufacturer's instruction.

2.4. Radioimmunoassay for corticosterone

Total plasma CORT levels were measured by radioimmunoassay using rabbit antiserum (antibody B3-163, Endocrine Sciences, Tarzana, CA) as previously described (Deak et al., 2003; Deak et al., 2005b). This antiserum was employed due to its low cross reactivity with other glucocorticoids and their metabolites. Assay sensitivity

was 0.5 $\mu\text{g/dl}$ (assay volume=20 μl plasma). The inter- and intra-assay coefficients were 13% and 10%, respectively.

2.5. Colorimetric assay for glucose

Plasma levels of glucose were measured using a commercially available kit (Stanbio Laboratory, Boerne, TX) based on the method of Trinder (1959) and adapted for use with a microtiter plate (Deak et al., 2005b). According to the manufacturer's instructions, this assay has linear sensitivity for glucose detection ranging from 0 to 500 mg/dl.

2.6. Statistical analysis

All analyses were conducted in Statview using an Analysis of Variance (ANOVA) appropriate to the study conducted (described individually below). Post hoc analyses were performed using Fisher's Protected Least Significant Difference (PLSD) method when an overall significant main effect was observed. Criterion for rejection of the null hypothesis was always $p < 0.05$.

2.6.1. Experiment 1: propranolol and footshock

The goal of this experiment was to determine whether propranolol (a pan β -adrenergic antagonist) would block footshock-induced increases in hypothalamic and splenic IL-1 β . Thus, rats ($n=8-9$ per group) were injected i.p. with 20 mg/kg of propranolol or equivalent volume of vehicle (doses based on Kohno et al. (1999)). Fifteen minutes later, rats were exposed to 80 intermittent footshocks (2.0 mA, 5 s each, 90 variable ITI) or remained in their home cage as non-stressed controls as in our previous work (Deak et al., 2003). Immediately after footshock, rats were sacrificed by rapid decapitation and tissue was harvested as described above.

2.6.2. Experiment 2: desipramine and footshock

The goal of this experiment was to assess whether desipramine (a relatively selective noradrenergic reuptake inhibitor) would facilitate the footshock-induced increase in hypothalamic and splenic IL-1 β . Thus, rats ($n=8-10$ per group) were injected s.c. with 20 mg/kg of desipramine or equivalent volume of vehicle. This dose was chosen because our laboratory (Deak et al., 2005c) and others (Cryan et al., 2002) have shown that this dose is behaviorally active in rats examined in the forced swim test, indicating clear CNS penetration and pharmacological activity. Thirty min later, rats were exposed to 80 intermittent footshocks (2.0 mA, 5 s each, 90 variable ITI) or remained in their home cage as non-stressed controls. Immediately after footshock, rats were sacrificed by rapid decapitation and tissue was harvested as described above.

2.6.3. Experiment 3: minocycline and footshock

Minocycline is a highly lipophilic tetracycline that crosses the blood–brain barrier easily (Aronson, 1980;

Colovic and Caccia, 2003; Zhanel et al., 2004) and exhibits anti-inflammatory properties that appear to be independent of the antimicrobial properties it possesses. Minocycline has been widely used to block central inflammatory responses and appears to have effects that are quite specific to actions on microglia (Raghavendra et al., 2003; Yrjanheikki et al., 1998; Yrjanheikki et al., 1999). We therefore hypothesized that administration of this selective microglial inhibitor would block hypothalamic but not splenic IL-1 β responses to footshock. To do this, rats ($n=8-9$ per group) were injected i.p. with 40 mg/kg of minocycline or equivalent volume of vehicle. This dose was chosen because Raghavendra and colleagues (Raghavendra et al., 2003) recently showed that 40 mg/kg i.p. completely abolished the expression of multiple pro-inflammatory cytokines in spinal microglia following nerve crush injury. One hour later, rats were exposed to 80 intermittent footshocks (2.0 mA, 5 s each, 90 variable ITI) or remained in their home cage as non-stressed controls. Immediately after footshock, rats were sacrificed by rapid decapitation and tissue was harvested as described above.

2.6.4. Experiment 4: minocycline effects on conditioned fear responding

One alternative explanation for the finding of Experiment 3 is that minocycline may have simply reduced the aversiveness of the stressor (due to non-specific sedative properties of the drug, etc), thereby decreasing the ability of footshock to induce a hypothalamic IL-1 β response. One way to assess the aversiveness of a stressor is to measure the amount of fear conditioned to the context in which the shock is delivered. Thus, as a preliminary test of this alternative hypothesis, we performed 2 experiments (4a and 4b). The goal of the first experiment was to ensure that our fear conditioning procedure was sensitive to the intensity of the footshock stimulus. In this experiment, rats ($n=4$ per group) were placed into the footshock chambers and given 2–3 min to acclimate to the context. Rats then received 2 footshocks (5 s each) separated by approximately 90 s that varied in intensity for each rat (ranging from 0 to 2.0 mA). Following a 5 min consolidation period, rats were returned to their home cage for 24 h, after which they were returned to the shock context for 15 min. During this test session, rats were videotaped from a downward angle for later measurement of conditioned fear (freezing) by observers blind to experimental treatment. Freezing was defined as the absence of all movement except for that necessary for normal respiration [as described in our previous work (Deak et al., 1999a)]. The number of 5 s intervals during which freezing was the dominant behavior were tabulated for the entire 15 min session. In the second experiment (4b), rats ($n=8$ per group) were injected with 0, 20, or 40 mg/kg of minocycline (i.p.) 60 min prior to both conditioning and testing sessions (performed as in Experiment 4a, with the 2.0 mA shock intensity). Fear conditioning was then assessed as described above.

3. Results

3.1. Experiment 1: propranolol and footshock

As in our previous work (Deak et al., 2003), intermittent footshock exposure significantly increased hypothalamic IL-1 β (a two-fold increase) relative to non-stressed controls. Importantly, this effect was completely blocked by pretreatment with propranolol [$F(1,28)=4.36$, $p<0.01$; see Fig. 1]. As expected, footshock also significantly increased splenic levels of IL-1 β [$F(1,28)=8.68$, $p<0.01$]. Interestingly, the footshock-induced increase in splenic IL-1 β was also reversed by pretreatment with propranolol [$F(1,28)=17.24$, $p<0.001$], indicating that activation of β -adrenergic receptors is necessary for stress-induced increases in both hypothalamic and splenic IL-1 β . Plasma concentrations of CORT [$F(1,28)=182.57$, $p<0.0001$] and glucose [$F(1,27)=20.43$, $p<0.001$] were significantly increased after footshock, but these effects did not vary across drug treatment groups [$F(1,28)=0.64$, $p>0.05$] and [$F(1,27)=0.98$, $p>0.05$], respectively. The lack of an effect of propranolol on the CORT response to footshock is consistent with previously published studies showing that norepinephrine induced activation of the pituitary–adrenal axis is mediated by α 1-adrenergic receptors and not β -adrenergic receptors (Plotsky

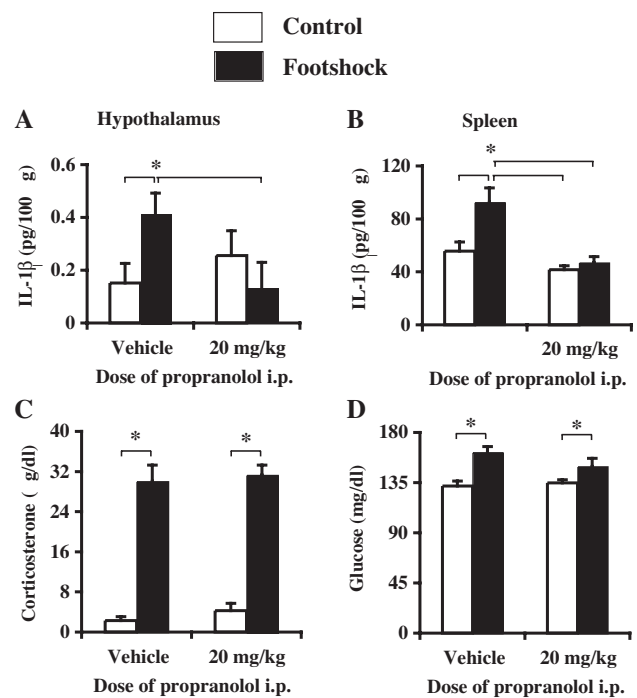


Fig. 1. Rats remained in home cages as non-stressed controls (white bars) or were given 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h (black bars). Rats were injected with 20 mg/kg of propranolol or equivalent volume vehicle 15 min prior to stressor exposure. Footshock significantly increased hypothalamic (panel A) and splenic IL-1 β (panel B) and this effect was reversed by propranolol. Plasma corticosterone (panel C) and glucose (panel D) were significantly increased after footshock. All data are expressed as mean \pm S.E.M. *Indicates significant difference between groups connected by bars ($p < 0.05$).

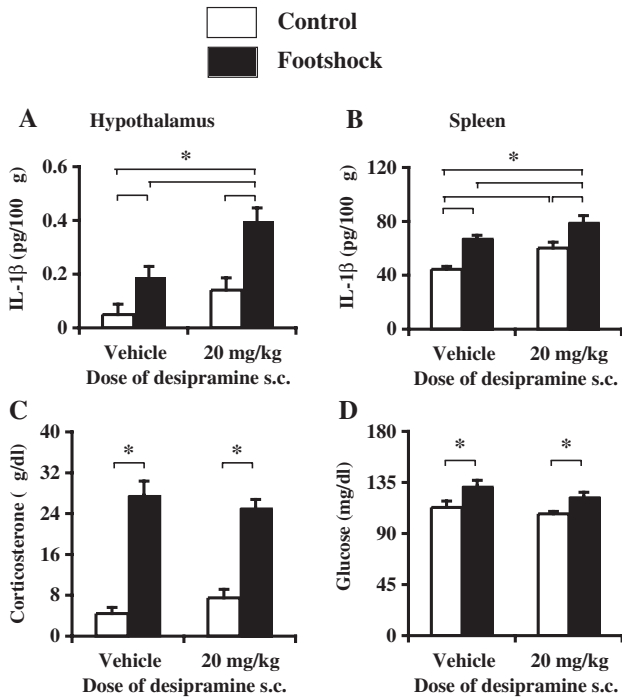


Fig. 2. Rats remained in home cages as non-stressed controls (white bars) or were given 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h (black bars). Rats were injected with 20 mg/kg of desipramine or equivolume vehicle 30 min prior to stressor exposure. Footshock significantly increased hypothalamic (panel A) and splenic IL-1β (panel B) and this effect was significantly facilitated by desipramine. Plasma corticosterone (panel C) and glucose (panel D) were both increased by footshock. All data are expressed as mean±S.E.M. *Indicates significant difference between groups connected by bars ($p < 0.05$).

et al., 1989). These data indicate that activation of β-adrenergic receptors was necessary for the footshock-induced increase in hypothalamic and splenic IL-1β.

3.2. Experiment 2: desipramine and footshock

Once again, footshock exposure significantly increased hypothalamic IL-1β (a two-fold increase) relative to non-stressed controls as indicated by a significant main effect of stress ($F(1,32)=15.84, p < 0.001$; see Fig. 2). Desipramine also significantly increased hypothalamic IL-1β levels relative to vehicle injected controls [$F(1,32)=8.88, p < 0.01$], and post hoc analyses revealed that baseline IL-1β levels were elevated after desipramine alone (relative to vehicle controls) and that the hypothalamic IL-1β response to footshock was significantly greater in the desipramine–footshock group than in the vehicle–footshock group. A similar pattern of results were observed in the spleen, where footshock again significantly increased splenic levels of IL-1β [$F(1,32)=42.17, p < 0.001$]. The footshock-induced increase in splenic IL-1β was also facilitated by pretreatment with desipramine [$F(1,32)=17.87, p < 0.001$]. Post hoc analyses revealed that splenic IL-1β levels were significantly increased in the desipramine–footshock group

relative to the vehicle–footshock group. Though plasma concentrations of CORT [$F(1,32)=106.95, p < 0.0001$] and glucose [$F(1,32)=13.14, p = 0.001$] were significantly increased after footshock, these effects did not vary across drug treatment groups [$F(1,32)=0.03, p > 0.05$] and [$F(1,32)=2.51, p > 0.05$], respectively. Together, these data support our hypothesis that catecholamines are critically involved in the hypothalamic and splenic IL-1β response to footshock.

3.3. Experiment 3: minocycline and footshock

This experiment tested whether inhibition of microglia by peripheral injection of minocycline would block the footshock-induced increase in hypothalamic IL-1β. Again, footshock exposure significantly increased hypothalamic IL-1β (a two-fold increase) relative to non-stressed controls and pretreatment with minocycline completely blocked the footshock-induced increase in hypothalamic IL-1β [$F(1,31)=19.58, p < 0.0001$; see Fig. 3]. Additionally, pretreatment with minocycline had no effect on the footshock-induced increase in splenic IL-1β [$F(1,31)=0.28, p > 0.05$], supporting the specificity of the pharmacological effects of minocycline on

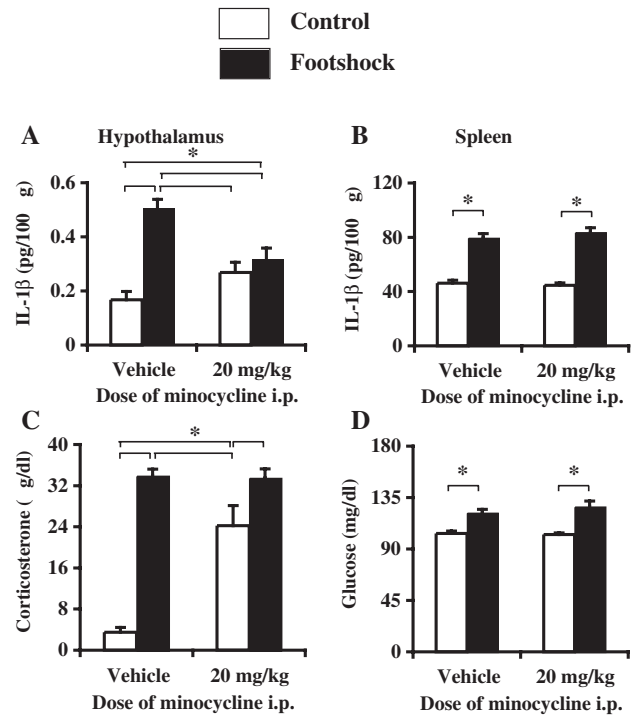


Fig. 3. Rats remained in home cages as non-stressed controls (white bars) or were given 80 inescapable footshocks (2.0 mA, 90 s variable ITI, 5 s each) over the course of approximately 2 h (black bars). Rats were injected with 40 mg/kg of minocycline or equivolume vehicle 1 h prior to stressor exposure. Footshock significantly increased hypothalamic (panel A) and splenic IL-1β (panel B) and only the hypothalamic IL-1 response was reversed by minocycline. Plasma corticosterone (panel C) and glucose (panel D) were both increased by footshock. All data are expressed as mean±S.E.M. *Indicates significant difference between groups connected by bars ($p < 0.05$).

microglia. Interestingly, while minocycline pretreatment had no effect on the CORT response in the footshock-exposed groups, basal levels of CORT were significantly elevated in the minocycline-injected controls relative to the vehicle injected controls [$F(1,31)=19.17$, $p<0.0001$]. This elevation in basal levels of CORT seen between the control groups and not between the footshock exposed groups led to a significant interaction [$F(1,31)=20.77$, $p<0.0001$], suggesting that minocycline alone elevated basal CORT levels. CORT levels were not significantly increased by minocycline in rats exposed to footshock. Footshock significantly increase glucose [$F(1,31)=48.79$, $p<0.0001$] and was unaffected by pre-treatment with minocycline [$F(1,31)=0.58$; $p>0.05$]. These results support the view that minocycline has actions specific to microglia and that microglia participate in the hypothalamic IL-1 β response to footshock.

3.4. Experiment 4: minocycline effects on conditioned fear responding

The total number of 5 s intervals during which fear conditioning was observed across the 15 min test session were analyzed using a one-way ANOVA. As expected, the amount of fear conditioned to the context varied significantly with shock intensity [$F(3, 12)=15.65$, $p<0.001$], and post hoc analysis indicated significant conditioned fear when the shock stimulus was set at 2.0 mA, but not 0.5 or 1.0 mA. Although a modest amount of freezing was observed when the shock stimulus was set at 0.5 or 1.0 mA, these effects were not significant when compared to the non-shocked controls (0 mA group). It should be noted that our shockers have an extremely long duty cycle, so comparison of shock intensities to our previous work (Deak et al., 1999a,b) should be made with caution. Importantly, these data indicate that our fear conditioning paradigm is sensitive enough to detect changes in conditioned fear responding, and justify the parameters used in Experiment 4b.

The goal of Experiment 4b was to determine whether minocycline might interfere with conditioned fear responding. Data were analyzed by one-way ANOVA and no effects of injection condition were observed [$F(2,27)=0.17$, $p>0.05$]. We therefore conclude that minocycline does not appear to alter the aversiveness of footshock, at least to the extent that fear conditioning can be used to assess the aversiveness of 2 footshocks (Fig. 4).

4. Discussion

The present data replicate our previous work showing that exposure to a 2 h session of intermittent footshock increased both hypothalamic and splenic IL-1 β concentrations (Deak et al., 2003). We now extend these findings mechanistically by showing that (i) pretreatment with the β -adrenergic antagonist propranolol completely blocked both

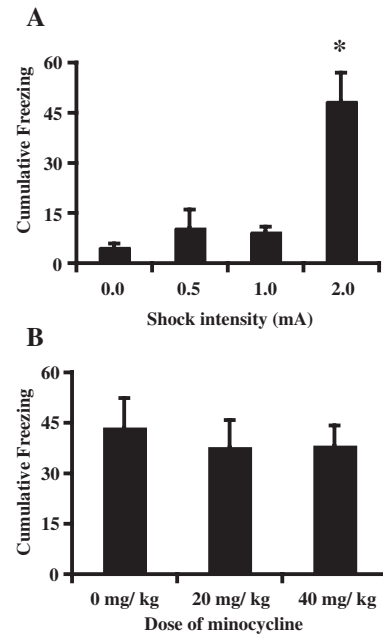


Fig. 4. A. Rats were exposed to 2 footshocks at the specified intensity and returned to their home cages. The amount of fear conditioned to the context was assessed the next day. Conditioned fear was only observed at a shock intensity of 2.0 mA. B. A separate group of rats was injected with vehicle or varying doses of minocycline (i.p.) prior to both conditioning and testing to assess the impact of the putative microglial inhibitor on fear conditioning. Pretreatment with minocycline had no effect on conditioned fear. All data are expressed as mean \pm S.E.M. *Indicates significant difference ($p<0.05$).

the hypothalamic and splenic IL-1 β responses to footshock; (ii) blockade of noradrenergic reuptake with desipramine facilitated basal as well as stress-precipitated increases in hypothalamic and splenic IL-1 β ; and (iii) the microglial inhibitor minocycline blocked the hypothalamic IL-1 β response to footshock, while the splenic IL-1 β response to footshock was completely unaffected in the same animals. Together, these data suggest that the availability of norepinephrine at adrenergic receptors appears to be critical for determining the magnitude of the IL-1 β response observed after footshock, at least in the two structures that we examined. Moreover, blockade of the footshock-induced increase in hypothalamic IL-1 β by the putative microglial inhibitor minocycline further suggests that the effects seen in the CNS may occur due to action at noradrenergic receptors on microglia. Additional studies examining the cellular localization of IL-1 β will be necessary to conclusively implicate microglia as the cellular source of IL-1 after footshock, and are the next logical step in this line of inquiry.

While we did not directly assess the specificity of action for minocycline in the present studies, previous work has established this agent as a highly selective method for inhibiting microglia (Raghavendra et al., 2003; Yrjanheikki et al., 1998; Yrjanheikki et al., 1999). The observation that minocycline blocked the hypothalamic IL-1 β response to

footshock but not the splenic IL-1 β response illustrates the specificity of minocycline. Importantly, minocycline has excellent penetration of the CNS when administered peripherally (Aronson, 1980; Zhanel et al., 2004) and appears to be especially capable of blocking pro-inflammatory cytokine production by microglia following various forms of traumatic injury (Ledeboer et al., 2005; Raghavendra et al., 2003), though its precise mechanism of action remains unclear (Zhanel et al., 2004). The fact that rats treated with minocycline behave quite normally in the forced swim test (Deak et al., 2005c) and show no apparent changes in fear conditioning (Experiment 4b) indicate that the action of minocycline in Experiment 3 is unlikely to be the result of nonspecific cognitive/perceptual alterations (sedation, etc.) produced by the drug. We can therefore tentatively conclude that microglia participate in the hypothalamic IL-1 β response to footshock, though the precise nature of microglial participation in this response remains to be determined.

One interesting question that arises from the present data is whether the splenic and hypothalamic IL-1 β responses simply co-occur in time or whether these effects depend on one another. Peripheral nerves such as the vagus (Goehler et al., 2000), sciatic (Herzberg and Sagen, 2001) and glossopharyngeal (Romeo et al., 2001, 2003) are thought to serve as one communication pathway between the peripheral immune response and the brain. In this sense, peripheral increases in cytokines can produce central increases in cytokines through activation of peripheral noradrenergic afferent nerves. A second communication pathway exists whereby peripheral immune signals on the blood side of the blood–brain barrier (Banks et al., 1991) stimulate immune signals on the brain side of the blood–brain barrier, possibly through action on endothelial cells (Quan et al., 2002). It is therefore possible that the effects observed in Experiment 3 are due to an impairment of immune-to-brain signaling through the neural or humoral routes just described. However, we don't consider this latter mechanism to be a critical concern for several reasons. First and foremost, though plasma cytokine levels were not analyzed in the present experiments, our previous work showed that plasma IL-1 β levels were not affected by the regimen of footshock to which rats were exposed in the present work (Deak et al., 2003), suggesting that blood contribution to the tissue content of IL-1 β is unlikely. Second, we have systematically compared IL-1 protein levels in the CNS from rats that were killed by rapid decapitation or after saline perfusion (Nguyen et al., 1998). No differences in central IL-1 were found between perfused and non-perfused tissue in those experiments, so it is unlikely that circulating cytokine contributes to the tissue content of IL-1, at least as far as the hypothalamus is concerned (spleen was not evaluated in our previous work). Thus, the increases seen in hypothalamic IL-1 β as a result of footshock appear to be centrally driven and not by blood-borne cytokine signaling. Lastly, recent work by Johnson

and colleagues (Johnson et al., *in press*) shows a clear dissociation between circulating and central IL-1 concentrations, and that IL-1 changes in these two compartments are mediated by separate adrenergic receptor mechanisms. While our data do not address these points directly, they do indicate that hypothalamic and splenic IL-1 responses to stress are separable phenomena, and that activation of noradrenergic receptors apparently underlies both effects.

In all 3 experiments, plasma concentrations of CORT and glucose were significantly increased by footshock. Propranolol and desipramine had no effect on either of these parameters although minocycline alone did. Considering compelling evidence that pituitary–adrenal activation by NE occurs via α 1-adrenergic receptors [e.g., (Plotsky et al., 1989)], it is not surprising that propranolol failed to alter the CORT response to footshock. One might have predicted a facilitation of the CORT response after desipramine, but considering that only a single time point for CORT was examined and ample time for feedback inhibition on the axis had lapsed, this null effect may not be particularly meaningful in and of itself. It may also be tempting to interpret that the minocycline-induced increase in basal CORT from Experiment 3 may account for the action of minocycline on hypothalamic IL-1 β , particularly since CORT inhibits IL-1 expression (Nguyen et al., 1998; Nguyen et al., 2000). However, we consider this to be an unlikely scenario because rats exposed to footshock exhibited identical CORT levels irrespective of whether vehicle or minocycline was administered prior to exposure. In addition, minocycline had no effect on the splenic IL-1 β response to footshock, even though the spleen is rich in corticosteroid receptor expression [e.g., Spencer et al., 1990]. Finally, CORT-mediated inhibition of cytokine production tends to occur at pharmacological (i.e., supra-physiological) doses (Munck et al., 1984), whereas lower concentrations of CORT have been shown to facilitate immune function (Dhabhar et al., 1996; Fleshner et al., 2002). Thus, the action of minocycline to block the hypothalamic IL-1 β response is unlikely to occur as a result of CORT-mediated suppression of IL-1 β .

Using circulating glucose as another marker of stressor reactivity (Armario et al., 1990), main effects of footshock were always observed with no effects of drug treatment. These findings were surprising because several reports suggest that hypothalamic noradrenergic pathways contribute to the central control of circulating glucose (Smythe et al., 1984, 1989) and our previous work has shown that regulation of circulating glucose may be an important factor in the generation of hypothalamic IL-1 β responses to stress (Deak et al., 2005b). Despite this discrepancy, CORT and glucose do not seem to account for the differences seen in hypothalamic and splenic IL-1 β responses to footshock, further supporting the direct role of the noradrenergic system.

To briefly summarize, our data suggest that activation of noradrenergic receptors is necessary for the observation of

stress-induced increases in hypothalamic and splenic IL-1 β , and that these effects occur independent of stressor-induced corticosterone and glucose reactivity. In addition, our data indicate that microglia may be a primary cellular source of IL-1 β in the CNS during times of stress and noradrenergic activation of microglia could provide a mechanism by which certain stressors (i.e. footshock) elicit priming-like effects towards later immune challenge (Deak et al., 2005a; Johnson et al., 2002; Johnson et al., 2003). Moreover, given the emerging involvement of pro-inflammatory cytokines and microglia in the development of neurodegenerative disorders, our data suggest that noradrenergic activation of microglia by stress may be one mechanism by which stress exacerbates and/or precipitates such deleterious processes in certain prone individuals.

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