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Deletion of the ventral noradrenergic bundle obliterates the early ACTH response after systemic LPS, independently from the plasma IL-1β surge

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We have recently shown that total lesion of the ventral noradrenergic bundle (VNAB-X), enhanced the short-lived (<120 min) triggering effect of intra-arterially (i.a.) given IL-1β on plasma ACTH levels. In the present study we used the same VNAB-X paradigm to explore the mechanisms of the long-lived (480 min) LPS stimulatory effect on plasma ACTH, corticosterone (CORT) and IL-1β levels. In control rats, 25 μg kg^{-1} LPS induced a 20-fold increase in ACTH and a 7-fold increase in CORT concentrations at 30 min, which continued to rise until 60 min, before receding to baseline at 480 min. In contrast, the plasma IL-1β concentration started to increase above undetectable levels only at 120 min. In VNAB-X animals, the early (30 min) ACTH/CORT response to LPS was completely blunted, and the ACTH surge was reduced by 75% at 60 min. However, the sustained hormonal response (120 to 480 min) was unaltered. Both the temporal pattern and the amplitude of the plasma IL-1β response were normal. We conclude that (1) the VNAB is involved in the early (first 60 min) ACTH/CORT response to systemic LPS, (2) plasma IL-1β does not appear to be associated with this early corticotropic activation and (3) the later stages of the ACTH/CORT response to LPS (60 to 480 min) appear to be independent of the VNAB control and may therefore involve different control mechanisms, in which the IL-1β, by this stage massively released in the blood, may play a major role.

Keywords: stress; ventral noradrenergic ascending bundle; IL-1beta; corticosterone; ACTH; lipopolysaccharide

Introduction

Lipopolysaccharide (LPS), a potent bacterial endotoxin, consistently activates the hypothalamic-pituitary-adrenocortical (HPA) axis, and several of the cytokines released in cascades after LPS challenge act as humoral intermediaries between the immune and the neuroendocrine systems (Besedovsky & DelRey, 1992; Rivier, 1993). Indeed, three cytokines, Interleukin-1-β (IL-1β), Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα) have repeatedly been reported to stimulate ACTH secretion [cf rev in (Tilders *et al.*, 1994)]. However, the precise role of the three ACTH-promoting cytokines on the HPA response to LPS remains unclear.

There has been much attention focused on IL-1β as a major factor for HPA recruitment within the wider framework of LPS-induced hormonal responses. Indeed, whether administered at the periphery (Sapolsky *et al.*, 1987) or directly into the paraventricular nuclei (PVN) (Barbanel *et al.*, 1990), IL-1β quickly triggers CRH41 release and 3 h later increases CRF mRNA levels in the PVN (Harbuz *et al.*, 1992). However, pretreatment with an IL-1 receptor antagonist (IL-1ra) has led to conflicting reports. Several studies reported that IL-1ra blocked the HPA response (Schotanus *et al.*, 1993) without affecting circulating IL-6 levels (Schotanus *et al.*, 1993), whereas others reported that IL-1ra-pretreatment only partially reduced the ACTH response to LPS (Dunn, 1992), as did pretreatment with an IL-1 antiserum (Rivier *et al.*, 1989). We recently showed that the early ACTH and corticosterone (CORT) responses (<60 min) to LPS challenge occurred well before any detectable increase in plasma TNFα, IL-1β and IL-6 concentrations (Givalois *et al.*, 1994). The question of whether and how blood-borne IL-1β contribute to the long-lived (8 h) HPA axis response to LPS remains unclear.

A series of recent studies have shown that, regardless of the route of IL-1β injection, the ventral noradrenergic ascending bundle (VNAB) innervating the PVN is involved in the HPA responses to the cytokine (Dunn, 1988; Kabiersch *et al.*, 1988; Weidenfeld *et al.*, 1989; Matta *et al.*, 1991; Chuluyan *et al.*, 1992; Barbanel *et al.*, 1990, 1993). A lesion restricted to the dorsal section of the VNAB more than halved the overall corticotropic response to IL-1β, whereas a lesion extending to the utmost ventral portions of the VNAB doubled the amplitude of the earliest stages of the HPA response to IL-1β (Barbanel *et al.*, 1993).

In the present study we used this unusual effect caused by larger VNAB lesions (VNAB-X) to explore the time course of LPS-induced ACTH and CORT surges, and the possible involvement of systemic IL-1β in this response.

Results

Effects of LPS in control rats

Fifteen min after injection of a single bolus of 25 μg kg^{-1} body weight (b.w.) LPS, ACTH release doubled (148 ± 40 pg ml^{-1} *vs* 47 ± 8 pg ml^{-1} at t_0, $P<0.05$), and continued to rise to 40-times above baseline at 60 min (2148 ± 230 pg ml^{-1}, $P<0.01$) before receding to 3-fold basal levels at 300 min, and ultimately back to baseline at 480 min. Vehicle injection alone in a control group had no measurable effect throughout the same sampling period (Figure 1A).

Measured in the same rats, the plasma CORT concentration rose from 30 min onwards to a maximum between 60 min and 120 min post-LPS (329 ± 19 ng ml^{-1} and 351 ± 19 ng ml^{-1} respectively *vs* 28 ± 2 ng ml^{-1} at t_0, $P<0.01$) (Figure 1B). CORT concentrations then returned to normal at 480 min. No CORT stimulation was detected after vehicle injection.

IL-1β was undetectable in the systemic circulation of controls and after vehicle injection (not shown). After LPS infusion, plasma IL-1β remained undetectable until 120 min, when its concentration surged to near maximal levels (1374 ± 626 pg ml^{-1} *vs* <80 pg ml^{-1} at t_0, $P<0.01$). The concentration of IL-1β remained high until 300 min after LPS challenge (902 ± 396 pg ml^{-1}, $P<0.05$), and decreased progressively thereafter (Figure 3).

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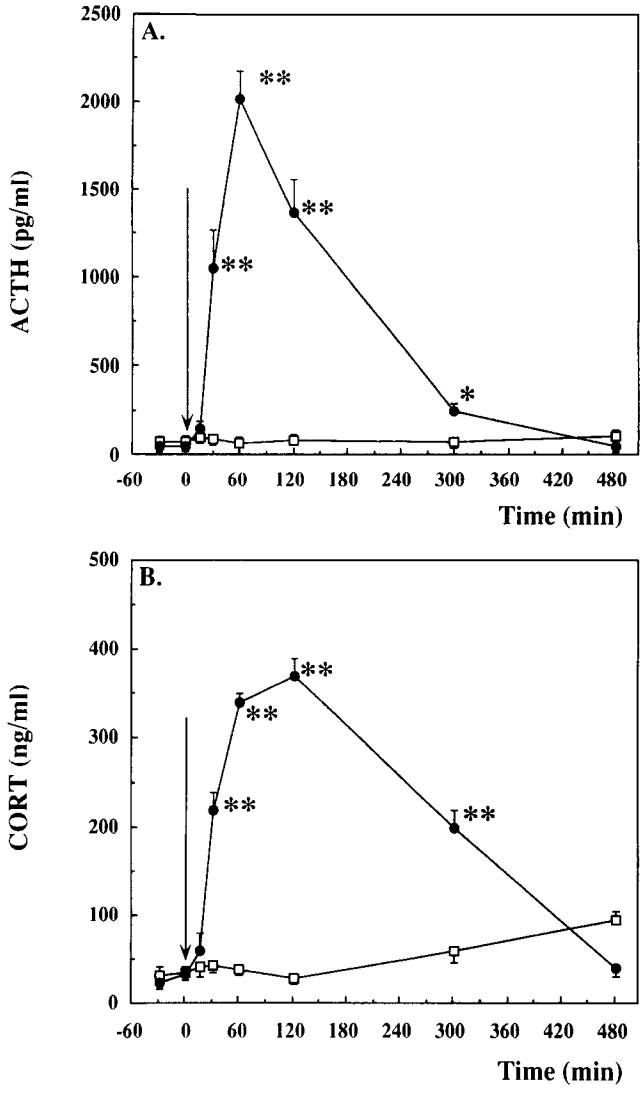

Figure 1 Temporal profiles of plasma ACTH (**A**) and corticosterone (CORT, **B**) after an intra-arterial infusion of either 25 μg kg^{-1} lipopolysaccharide (LPS) (●; $n = 8$) or saline (□; $n = 5$) into control rats. Means ± SEM. See text for the significance of the increases versus basal pre-injection (t-30 and t_0) levels in each group. *$P<0.05$ and **$P<0.01$ *vs* corresponding saline for the same time point

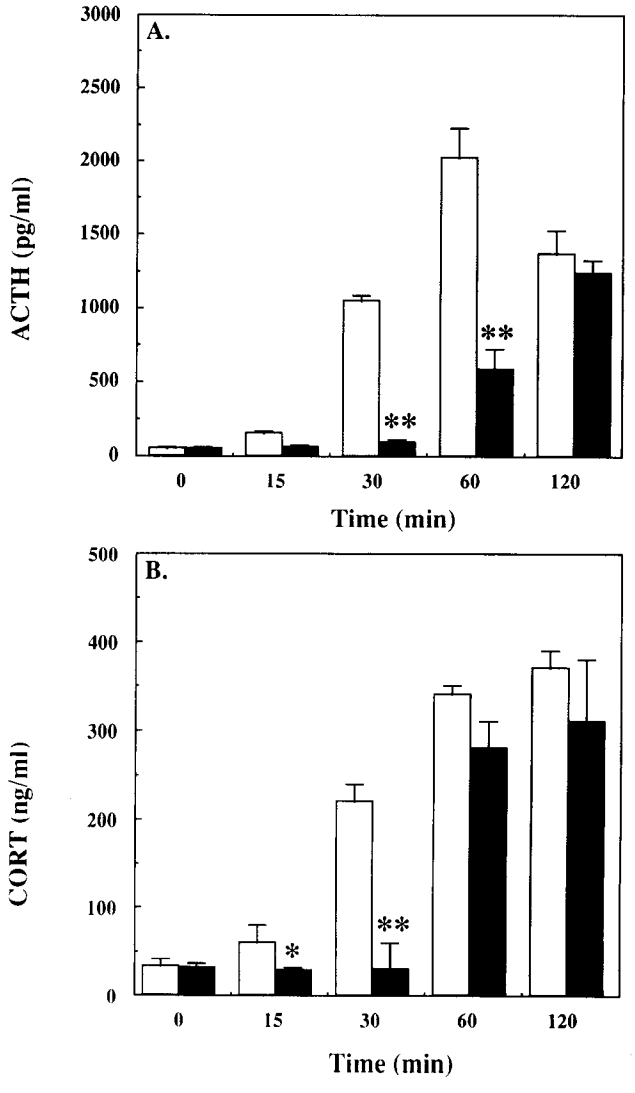

Figure 2 Variations of plasma ACTH (**A**) and corticosterone (CORT, **B**) after an intra-arterial infusion of 25 μg kg^{-1} lipopolysaccharide (LPS) injected into controls (gray bars; $n = 8$) and into rats bearing bilateral and complete lesions of the ventral noradrenergic ascending bundle (VNAB-X) (black bars; $n = 5$). Means ± SEM. See text for the significance of the increases versus basal pre-injection (t-30 and t_0) levels in each group. *$P<0.05$ and **$P<0.01$ *vs* corresponding controls for the same time point

Effect of LPS in VNAB-X animals

LPS injection in VNAB-X rats resulted in a pattern of ACTH secretion significantly different to that in control animals (ANOVA: F 1,11 = 11.4; $P<0.01$). For up to 60 min (Figure 2A), plasma ACTH levels were strikingly reduced (60 ± 4 pg ml^{-1} *vs* 148 ± 40 pg ml^{-1} in controls, $P>0.05$ at 15 min; 88 ± 19 pg ml^{-1} *vs* 1050 ± 213 pg ml^{-1}, $P<0.01$ at 30 min; and 582 ± 143 pg ml^{-1} *vs* 2148 ± 230 pg ml^{-1}, $P<0.01$ at 60 min). However from 120 min up to the end of the experiment (480 min), the ACTH patterns in lesioned and control rats were similar.

In contrast to plasma ACTH, the general pattern of CORT release was not significantly different in VNAB-X and control rats (ANOVA: F 1,11 = 3.4; NS). However, at the earliest post-LPS stages plasma CORT concentrations in VNAB-X animals were lower than the controls: 24 ± 1 ng ml^{-1} *vs* 70 ± 22 ng ml^{-1} at 15 min; ($P<0.05$) (Figure 2B) and 46 ± 21 ng ml^{-1} *vs* 229 ± 13 ng ml^{-1} at 30 min; ($P<0.01$). Thereafter, there was no significant difference between the two, in spite of the consistently slightly depressed CORT values measured in the VNAB-X animals, between 60 min (276 ± 31 ng ml^{-1} *vs* 329 ± 19 ng ml^{-1}) and 300 min (146 ± 55 ng ml^{-1} *vs* 220 ± 39 ng ml^{-1}).

The plasma profiles of IL-1β in LPS-injected VNAB-X animals were almost indistinguishable from those measured in unlesioned controls (ANOVA: F 1,9 = 0.17; NS) (Figure 3). In both groups IL-1β was undetectable until a sudden peak at 120 min (1166 ± 366 pg ml^{-1} in the VNAB-X group vs 1374 ± 626 pg ml^{-1} in controls). Thereafter the IL-1β values returned slowly and similarly to baseline (480 min), even though, the lesioned rats still displayed slightly but non significantly lower IL-1β levels than controls (244 ± 155 pg ml^{-1} *vs* 646 ± 375 pg ml^{-1}; NS).

Discussion

A large body of literature has recently accumulated describing the complex mechanisms underlying the integrated neuroendocrine/immune response to infectious aggressions (Besedovsky & DelRey, 1992; Rivier, 1993). Many questions have been raised in this rapidly expanding research field. We investigated one of these issues: the possible central nervous system involvement in the HPA response to an intra-arterial (i.a.) infusion of the bacterial endotoxin LPS. In control rats,

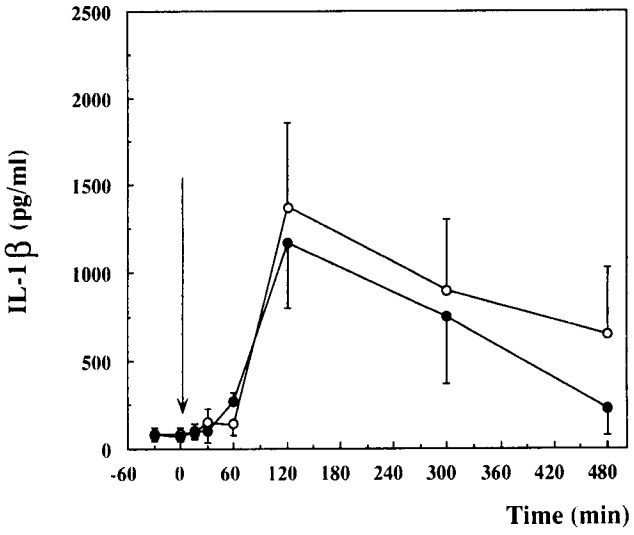

Figure 3 Temporal profiles of plasma Interleukin-1β (IL-1β) after an intra-arterial infusion of 25 μg kg^{-1} lipopolysaccharide (LPS) injected into controls (●; $n = 8$) and into rats bearing bilateral and complete lesions of the ventral noradrenergic ascending bundle (VNAB-X) (○; $n = 5$). Means ± SEM. See text for the significance of the increases versus basal pre-injection (t-30 and t_0) levels in each group

i.a. LPS induces an early (15 min) and long-lasting (>6 h) rise in plasma ACTH and CORT levels (Givalois *et al.*, 1994) and an increased release of CRH41 in push-pull cannulated median eminence is associated with the earliest stages of the ACTH/CORT response (Givalois *et al.*, 1993). We used the neurotoxic deletion of the catecholaminergic innervation of the PVN (VNAB-X), because a consensus has emerged that catecholamines play a major role in the HPA stimulation by a variety of stressors (reviewed in Gaillet *et al.*, 1993). We report that bilateral deletion of the VNAB expanding down to its most ventral portions blunted the earliest phase of the ACTH surge (first 60 min), whereas the later stages of the hormonal response (120 to 300 min) were unaffected. Thus, there appear to be at least two different mechanisms involved in the long-lived HPA response to exogenous LPS. Our findings are consistent with earlier studies showing that bilateral deletion of the parvocellular PVNs hosting the CRH41 neurons obliterated the early (60 to 90 min), but not the later stages of the ACTH response to LPS (Elenkov *et al.*, 1992). The first conclusion is that the CRH-producing neurons and their innervation by the VNAB are irreplaceable for the rapid recruitment of the HPA axis following an LPS challenge.

Regarding the triad of ACTH-activating cytokines: TNFα, IL-1β and IL 6 released after an LPS challenge (Besedovsky & DelRey, 1992; Rivier, 1993; Givalois *et al.*, 1994), most attention has been given to IL-1β as a major factor involved in the HPA recruitment, because peripheral or intra-PVN administration of the cytokine triggers CRH41 and ACTH releases within minutes (Sapolsky *et al.*, 1987; Barbanel *et al.*, 1990), and CRH41 mRNA levels in the PVN a few hours later (Harbuz *et al.*, 1992). However, various data are not in favor with IL-1β alone being responsible for the rapid and sustained HPA axis response to LPS: (i) the ACTH/CORT response to LPS is not fully blocked by either an anti-IL-1β antibody, or an IL-1β receptor antagonist pretreatment (Rivier *et al.*, 1989; Dunn, 1992; Perlstein *et al.*, 1993); (ii) the early ACTH/CORT response to systemic LPS (15 to 30 min) starts well before the large increase in plasma IL-1β levels (between 60 and 120 min) Givalois *et al.*, 1994). This casts doubt on the participation of plasma IL-1β in the early 20-fold increase in ACTH concentration.

It may, however, be argued that there are biologically efficient increases in circulating plasma IL-1β below the assay's sensitivity threshold (80 pg ml^{-1}) before 60 min, and that they contribute to the early activation phase of the HPA axis. However, we have shown in a previous study that an i.a. bolus of 100 ng IL-1β, which considering a blood/weight ratio of 7% may result in plasma IL-1β concentrations of about 4–10 ng ml^{-1}, only ellicited a moderate ACTH increase (400 pg ml^{-1}) (Barbanel *et al.*, 1993). The minimal effective dose of IL-1β on ACTH secretion was reported to be 0.1 mg kg^{-1}, corresponding to 1–2 ng ml^{-1} circulating IL-1β (Rivier & Shen, 1994). It seems therefore unlikely that in our model undetectable amounts of IL-1β in the plasma cause the ACTH surge before the large increase in plasma IL-1β, 2 h post-LPS.

Our present study affords another argument against plasma IL-1β promoting the primary phase of the ACTH post-LPS surge. Indeed, the total VNAB lesion used here has repeatedly been shown to block strongly the CRH41 and ACTH responses to stimuli such as ether or immobilization (Gaillet *et al.*, 1991, 1993). However, it was also shown to stimulate the short-lived ACTH surge (<120 min) when i.a. IL-1β was used as a stressor (Barbanel *et al.*, 1993). Quite unexpectedly, in the present study we observed that this type of VNAB lesion induced a near complete inhibition, restricted to the early (<60 min) phase of ACTH stimulation. The comparison of the opposite effects of large VNAB lesions in the ACTH response to either i.a. IL-1β or i.a. LPS strongly favors the idea that the primary phase of the long-lived ACTH response to LPS – which incidentally lasts the same time (60 min) as the whole response after IL-1β – may be activated independently of the blood borne IL-1β. Clearly, were the slightest increase in plasma IL-1β, be it under the detection level of our assay (IRMA), involved in this post-LPS response, VNAB-X might lead to potentiation rather than depression of the ACTH response to LPS.

Contrary to its effects on the earliest hormonal response to LPS, the complete VNAB lesion did not significantly affect the delayed (>60 min) and long lasting (300 min) ACTH/CORT and IL-1β responses to LPS (this study), nor impair the latest phase (60–120 min) of the short-lived ACTH/CORT surge after i.a. IL-1β (Barbanel *et al.*, 1993).

We conclude that (1) the initial HPA response to LPS involves CNS mechanisms which may be bypassed during the later stages of the response, and (2) the primary phase of the response only, is independent of plasma IL-1β levels. However, this does not rule out the possible participation of IL-1β as an intermediary signal between systemic LPS and the HPA axis, as IL-1β was shown to be ubiquitous, and LPS appears to increase IL-1β production in the brain (Hillhouse & Mosley, 1993; Kakucska *et al.*, 1993). However, hypothalamic IL-1β was not detected before one hour after LPS challenge (Hagan *et al.*, 1993) and the putative link between its cerebral production and the early HPA axis stimulation remains to be demonstrated. In an indirect approach to this question we recently showed that even minute amounts of IL-1β directly infused into the PVN produced an almost immediate stimulation of CRH41 and ACTH releases (Barbanel *et al.*, 1990), and that this response was blunted by total VNAB lesion (Barbanel *et al.*, 1993). This may be compared with the strongly depressed ACTH response within the first hour post-LPS described above. Overall, the data indicate that a variety of different mechanisms are apparently set into action for humoral stressors – e.g. IL-1β or LPS – produced endogenously or administered experimentally at different sites, e.g. locally at the periphery, or in the brain, or blood borne (Rivest *et al.*, 1992; Barbanel *et al.*, 1993; Kakucska *et al.*, 1993; Wan *et al.*, 1993).

Alternatively, IL-1β release in peripheral tissues or LPS itself may act locally on nocireceptors (Bluthé *et al.*, 1992), and the stimuli thus generated may be ultimately conveyed by the VNAB to the CRH41-producing neurons, as one of the major common pathways for the recruitment of the HPA axis in response to a variety of endogenous and exogenous aggressive stimuli (Assenmacher *et al.*, 1987, 1992, 1994).

To sum up (1) the VNAB is strongly involved in the early

(first 60 min) but not in the longer-lived (300 min) ACTH/CORT response to systemic LPS; (2) plasma IL-1β does not appear to be associated with this early corticotropic response, because (i) the cytokine's plasma levels remained below its IRMA detection level before rising rapidly to 16-fold the detection level at 120 min and (ii) the primary phase of the ACTH response which was strongly depressed by a wide VNAB lesion, corresponds precisely to the time sequence where, for the same lesion, to stimulation and not inhibition of the ACTH response to systemic IL-1β was earlier shown; (3) the later stages of the ACTH/CORT response to LPS (60 to 300 min) appear independent of VNAB control, and may involve different regulatory mechanisms, in which the ACTH-stimulating cytokines, then massively released into the blood stream, may play a major role.

Materials and methods

Animals

Male Sprague Dawley rats (DEPRE, St Doulchard, France) weighing 280–300 g at the beginning of the experiment, were individually caged in a sound proof facility with controlled temperature (21 ± 1°C) and lighting regimen (12 h light starting at 7 a.m.). Commercial rat chow and tap water were provided *ad libitum*. Rats were implanted under deep Equi-Thesin anesthesia (4 ml kg^{-1}) with an indwelling carotid cannula as previously described, to allow both intra-arterial (i.a.) injections and subsequent sequential blood sampling (Barbanel *et al.*, 1993). During the same surgical session, the VNAB was bilaterally and totally deleted by administration of 6-hydroxydopamine (6-OHDA, 10 μg in 0.5 μl solvent) at the stereotaxic coordinates previously used and reported (Barbanel *et al.*, 1993). The histological and neurochemical conformity of the lesions to these standards was checked post-mortem. Control rats were injected with the solvent alone. During the 5 day recovery period, the animals were handled twice daily, to adapt them to the experimental conditions and blood samples were collected daily and replaced with heparinized saline.

On the day of the experiment, a series of individual blood samples was taken, starting at 8.30 a.m. to minimize an interaction with the HPA circadian rhythm. Samples (300 μl) were collected on EDTA from all rats, 30 min and immediately before injection (t_0), and 15, 30, 60, 120, 300 and 480 min thereafter. Lipopolysaccharide (LPS, from *E. coli* 055:B5; Sigma), dissolved in 500 μl kg^{-1} b.w. saline, was infused over 15 s at a dose of 25 μg kg^{-1}. This dose was chosen according to an earlier dose-response study (Givalois *et al.*, 1994). After collection, samples were centrifuged at 4°C, and plasma was stored with 5 μl Aprotinin at −30°C until assay. Immediately after the last sampling, 8 h post-LPS, the rats were decapitated, their brains removed, the hypothalami were quickly dissected out, frozen in liquid nitrogen and stored at −70°C until measurement of noradrenaline.

Ethics

Conforming to French Laws on Laboratory animals, the experimental protocol was agreed by the Directory Committee of the University's 'Center for Laboratory Animals'.

Hypothalamic concentration of noradrenaline

To check the efficacy of the VNAB lesions to deplete the hypothalamus in noradrenaline, we used the same method as in our previous studies, which incidentally showed that the effects were similar when NA concentrations were measured either in hypothalamic extracts or in PVN punches (Gaillet *et al.*, 1993). Briefly, the tissue samples were homogenized by ultrasonication in ice-cold 0.1 M $HClO_4$, immediately after thawing. After centrifugation, 20 μl extracts were injected directly onto a C-18 reverse phase analytical column (Beckman ODS-IP) maintained at 30°C. The mobile phase consisted of 0.1 M phosphate/citrate buffer (pH = 3.5) containing 0.1 mM EDTA, 1 mM octylsulfonic acid, 2 mM triethylamine and 10 to 14% of a mixture of methanol/acetonitrile (1/1). Concentrations of NA were determined using a Chromatofield amperometric detector, set at 0.72 V relative to an Ag-AgCl reference electrode. For each sample, the peak area (Shimadzu CR 3A integrator) was compared with those of standards run in parallel. The sensitivity of the method for NA was 0.1 pmole/20 μl sample, corresponding to 0.5 pmole mg^{-1} protein (Coomassie Blue assay). Conforming to our previous studies, the extensive VNAB lesion used led to a 80% fall in the hypothalamic NA contents as compared to sham injected controls and i.a. LPS itself slightly (−15 to −20%) depressed hypothalamic NA in both intact controls and VNAB lesioned animals (Table 1).

Table 1 Effects of LPS challenge on hypothalamic Noradrenaline contents of controls or VNAB lesioned rats

	Vehicle	*LPS*
Control	132 ± 10	112 ± 14
VNAB-X	26 ± 2**	20 ± 4**

Animals were subjected to a stereotaxic micro-injection of either vehicle or 6-OHDA, 5 days before being i.a. challenged with saline or 25 μg kg^{-1} lipopolysaccharide (LPS) as described in text. At the end of the plasma sampling (8 h after LPS), the animals were sacrificed and their hypothalamus dissected. Hypothalamic noradrenaline was measured by HPLC followed by an electrochemical detection. Means ± SEM (pmole mg^{-1} protein). $^{**}P<0.01$ *vs* control rats according to ANOVA.

Hormone and cytokine assays

ACTH levels were measured in 50 μl plasma samples using a RIA kit (ACTH K-PR; ORIS, Saclay, France) as previously described (Barbanel *et al.*, 1990, 1993). The assay's sensitivity threshold was 10 pg ml^{-1}, and intra- and inter-assay variation coefficients were 5% and 6%, respectively. CORT was assayed in 25 μl samples, using a radio-competition method (Barbanel *et al.*, 1993; Givalois *et al.*, 1994) with a sensitivity of 5 ng ml^{-1}, and intra- and inter-assay variation coefficients of 3% and 4%, respectively.

Plasma Interleukin-1β levels were estimated with the species-specific immunoradiometric assay recently described for rat IL-1β (Bristow *et al.*, 1991) and slightly modified (Givalois *et al.*, 1994). Briefly, 50 μl plasma samples were incubated in PVC multi-well plates previously coated with a specific polyclonal sheep anti-rat IL-1β antiserum, incubated for 24 h at 4°C before adding an excess of purified radio-iodinated anti-rat IL-1β antiserum. After a second 24 h incubation at 4°C, the plates were washed and the remaining radioactivity counted, and compared to a standard curve obtained with known doses of rat recombinant IL-1β. All samples were run in the same assay. The assay's sensitivity was 80 pg ml^{-1}. The intra- and the inter-assay coefficients of variations were 3% and 5% respectively.

By convention, for each measurement, samples containing undetectable amounts were allotted the sensitivity threshold of the assay, which obviously results in an underestimation of comparative stimulatory effects (Barbanel *et al.*, 1990). Concentrations are indicated as means ± SEM. For statistical analysis, we used a computer assisted ANOVA program followed by an F test.

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