Paraventricular–coerulear interactions: role in hypertension induced by prenatal undernutrition in the rat

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Abstract

Rats submitted to fetal growth retardation by *in utero* malnutrition develop hypertension when adult, showing increased hypothalamic mRNA expression for corticotropin-releasing hormone (CRH) and increased central noradrenergic activity. As hypothalamic CRH serves as an excitatory neurotransmitter within the locus coeruleus (LC) and coerulear norepinephrine plays a similar role within the paraventricular nucleus (PVN) of the hypothalamus, we studied, in both normal and prenatally undernourished 40-day-old anesthetized rats, the effects of intra-LC microinjection of CRH and intra-PVN microinjection of the α_1-adrenoceptor antagonist prazosin on multiunit neuronal activity recorded simultaneously from the two nuclei, as well as the effects on systolic pressure. Undernutrition was induced during fetal life by restricting the diet of pregnant mothers to 10 g daily, whereas mothers of control rats received the same diet *ad libitum*. At day 40 of postnatal life: (i) undernourished rats showed increased neuronal activity in the PVN and LC, as well as increased systolic pressure; (ii) intra-LC CRH stimulated LC and PVN neurons and increased systolic pressure only in normal rats; (iii) intra-PVN prazosin decreased LC and PVN neuronal activity and systolic pressure only in undernourished rats; and (iv) in normal rats, prazosin prevented the stimulatory effect of CRH only in PVN activity; in undernourished rats, prazosin allowed CRH to regain its stimulatory effects. The results point to the existence of an excitatory PVN–LC closed loop, which seems to be hyperactive in prenatally undernourished rats as a consequence of fetal programming; this loop could be responsible, in part, for the hypertension developed by these animals.

Introduction

Corticotropin-releasing hormone (CRH), a 41-amino-acid peptide synthesized mainly in neurons of the paraventricular nucleus (PVN) of the hypothalamus, functions as a neurotransmitter in some extrahypothalamic areas of the brain, such as the noradrenergic nucleus locus coeruleus (LC). Thus, CRH fibers (Swanson *et al.*, 1983; Sakanaka *et al.*, 1987; Valentino *et al.*, 1992), binding sites for CRH (Wynn *et al.*, 1984; Sanchez *et al.*, 1999) and synaptic contacts between CRH-immunoreactive terminals and dendrites (Van Bockstaele *et al.*, 1996) have been visualized in the LC. In addition, CRH administered intra-cerebroventricularly (Valentino *et al.*, 1983; Valentino & Foote, 1988) and directly into the LC (Page & Abercrombie, 1999) increased LC discharge rates, and increased the expression of Fos in the LC (Rassnick *et al.*, 1998). On the other hand, it has been pointed out that central norepinephrine stimulates magnocellular CRH-synthesizing neurons in the PVN. Thus, immunofluorescence and immunohistochemical studies have demonstrated axonal projections from the A1, A2, A6 (LC) and A7 noradrenergic groups to the magnocellular and parvocellular regions of the PVN (Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988). Electrical stimulation of these brainstem–PVN connections excited the majority of PVN neurons, an effect that is counteracted by 6-hydroxydopamine or the α_1-antagonist ergotamine and imitated by the α_1-adrenoceptor agonist phenylephrine (Day *et al.*, 1984; Tanaka *et al.*, 1985; Kim *et al.*, 1989; Saphier, 1989, 1993; Saphier & Feldman, 1991). As a whole, the above data point to the existence of an excitatory closed loop of reciprocally interconnected PVN and LC neurons, as has been suggested elsewhere (Dunn & Berridge, 1990; Dunn *et al.*, 2004).

The prenatal environment can modify the postnatal physiology (fetal programming) through adaptive changes in gene expression patterns that occur in response to stressors such as malnutrition, hypoxia or increased glucocorticoid exposure during early life. For example, fetal growth retardation by *in utero* malnutrition can induce hypertension in animals (Langley-Evans *et al.*, 1996a,b; Perez *et al.*, 2002) and humans (Barker *et al.*, 1989; Barker, 1992) when adult, which is mainly the result of enhanced systolic pressure. Increased CRH mRNA expression in the PVN of spontaneously hypertensive rats (Krukoff *et al.*, 1999), in the hypothalamus of prenatal undernutrition-induced hypertensive rats (Perez *et al.*, 2004) and in the PVN of humans with primary hypertension (Goncharuk *et al.*, 2002) has been reported, pointing to an important role for the PVN in the pathogenesis of hypertension in humans as well as in hypertensive states developed in animals.

To functionally characterize the reciprocal excitatory connections between PVN and LC neurons in both normal and prenatally undernourished young rats, we recorded simultaneously the multiunit

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neuronal activity in these nuclei and tested the effect of microinjecting CRH into the LC and/or the α_1-adrenoceptor antagonist prazosin into the PVN. The CRH microinjection into the LC allowed activation of the excitatory PVN–LC closed loop, whereas prazosin microinjected into the PVN allowed opening of the loop. In order to correlate the changes in neuronal activity with changes in cardiovascular function, the systolic blood pressure was assessed in animals subjected to similar pharmacological manipulations.

Materials and methods

Animals

The experimental protocol and animal management were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and were approved by the Committee for the Ethical Use of Experimental Animals, Institute of Nutrition and Food Technology, University of Chile. The experiments were carried out on male and female Wistar rats (Institute of Nutrition and Food Technology, Santiago, Chile), born from mothers subjected during pregnancy to one of the following nutritional conditions: (i) normal pregnant rats, with free access to a 21% protein standard laboratory diet (Champion, Santiago, Chile) and (ii) undernourished pregnant rats, with restricted access (10 g daily) to the standard laboratory diet throughout pregnancy; this amount of food is about 40% of that consumed by normal pregnant rats (Soto-Moyano *et al.*, 1998a). To prevent undernutrition of pups during postnatal life, prenatally undernourished pups were fostered at birth to well-nourished dams giving birth on that day, according to previously described rearing procedures (Morgane *et al.*, 1993). Pups born from well-nourished dams were nursed by their own mothers. During the lactation period all litters were adjusted to eight pups per dam and all dams continued to receive the standard laboratory diet *ad libitum*. After weaning at 22 days of age, all pups were housed eight per cage and fed on the standard laboratory diet. The body weights of pregnant mothers and pups were measured daily.

Electrophysiological recording and microinfusion of drugs

We used two double semi-microelectrode/micropipette assemblies inserted, respectively, into the LC and PVN of both eutrophic control and prenatally undernourished 40-day-old rats. This procedure allowed the simultaneous recording of the LC and PVN multiunit discharges and also allowed the microinfusion of known concentrations of CRH agonists into the LC and adrenergic antagonists into the PVN, to precisely characterize the effects of such ligands on reciprocal excitatory PVN–LC neuronal connections. The procedure used for drug microinfusion into a particular neuronal nucleus and simultaneous recording of its multiunit neuronal discharge in anesthetized rats has been reported elsewhere (Perez *et al.*, 1998). At 40 days of age, rats were anesthetized with 1.2 g/kg urethane and placed in a stereotaxic apparatus for rats (ST-7, Narishige Scientific Instrument Laboratory, Tokyo, Japan). The horizontal zero plane of the stereotaxic apparatus was tangent to the upper incisor bar and 5 mm above the inter-aural line. The body temperature was maintained at 37–38 °C with a small heating pad and monitored with a temperature probe connected to a digital thermometer inserted 2 cm into the rectum. The skull was exposed and a 3.0-mm diameter hole, centred at 1.0 mm lateral to the midline and 7.6 mm caudal to the bregma point, was drilled over the right occipital pole for approaching the LC according to the stereotaxic atlas of the rat brain of Pellegrino *et al.* (1979). A second 3.0-mm diameter hole, centred at 0.5 mm lateral to the midline and 0.6 mm rostral to the bregma point, was drilled in the right parietal bone for approaching the PVN. The dura was carefully removed using fine forceps and iridectomy scissors. Thereafter, a parylen-coated tungsten semi-microelectrode (20-μm tip, 0.3–0.5 MΩ) glued to a glass micropipette with a 20-μm tip (the tip of the micropipette was 0.3 mm rostral to the tip of the semi-microelectrode) was advanced to the LC at coordinates A, −1.6; L, 1.0 and V, −2.8 (in mm). A second semi-microelectrode/micropipette pair was advanced to the PVN at coordinates A, 6.4; L, 0.5 and V, −1.6 (in mm). The stereotaxic coordinates were taken from Pellegrino *et al.* (1979) and all coordinates are referred to the recording semi-microelectrode. As the brains of the prenatally undernourished animals are slightly but consistently smaller than those of eutrophic controls, coordinates for the semi-microelectrode/micropipette pair positioned in the PVN of undernourished rats were displaced 0.05 mm backwards and 0.02 mm downwards. No adjustment was needed for the semi-microelectrode/micropipette pair positioned in the LC. The multiunit activities recorded from the LC and PVN were amplified (two Grass P511 amplifiers, 0.1–3.0-kHz bandwidth), displayed on a dual-trace digital oscilloscope (3365A, Philips, Amsterdam, The Netherlands), digitized at 10 kHz by an analog/digital converter card interfaced to a microcomputer (Pentium PC, Acer Inc., Taipei, Taiwan), full-wave rectified and integrated in a time-window (hardware and software developed by the engineer Mr Lincoln Rivas, Santiago, Chile), and stored on hard disk for later analysis. The effect of CRH microinjected into the LC and/or the effect of the α_1-adrenoceptor antagonist prazosin microinjected into the PVN on the spontaneous multiunit neuronal activity simultaneously recorded from the LC and PVN were studied. In each experiment, three consecutive samples of multiple unit activity, of 2 s each, were recorded simultaneously from both nuclei before and 2, 5, 10, 15, 20, 25 and 30 min after drug microinjection. These samples were full-wave rectified, and then integrated and stored on hard disk. All drugs were from Sigma (St Louis, MO, USA) and were mechanically microinjected in a known volume (0.05 μL) with an electric-driven microinfusion pump (Stoelting Co., Wood Dale, IL, USA) under visual control (10 × stereoscopic magnifier) of the displacement of an air bubble left in the micropipette. Injection of the entire volume required 1–2 min. In both normal and prenatally undernourished rats, the experimental series included: (i) a baseline recording of multiunit activity from the LC and PVN; (ii) recording of multiunit activity from the LC and PVN after microinjection of 0.5 μg CRH into the LC; (iii) recording of multiunit activity from the LC and PVN after microinjection of 0.5 μg CRH into the LC, with previous (10 min) microinjection of 1.2 μg prazosin into the PVN; and (iv) recording of multiunit activity from the LC and PVN after microinjection of 0.05 μL of artificial cerebrospinal fluid (CSF) into the LC and PVN. Experimental series (ii), (iii) and (iv) were carried out in different groups of normal or undernourished animals but were always preceded by step (i). Recordings lasted for 30 min after microinjection of CRH or saline into the LC. The volume and doses of CRH and prazosin were taken from the literature (Ku *et al.*, 1998; Camargo & Saad, 2001). At the end of each experiment, the rat was killed with an overdose of urethane and samples of the background electrical noise recorded from the two nuclei were stored. This procedure allowed the subtraction of background noise from the recorded neuronal activity in each nucleus. Thereafter, the locations of both semi-microelectrode/micropipette pairs were marked with electrolytic lesions by passing a 1-mA cathodal current for 20 s in the LC and PVN. Animals were then perfused with intra-cardiac 10% formalin solution, and the brains were removed and processed histologically for verification of electrode placement (Fig. 1). Only experiments where

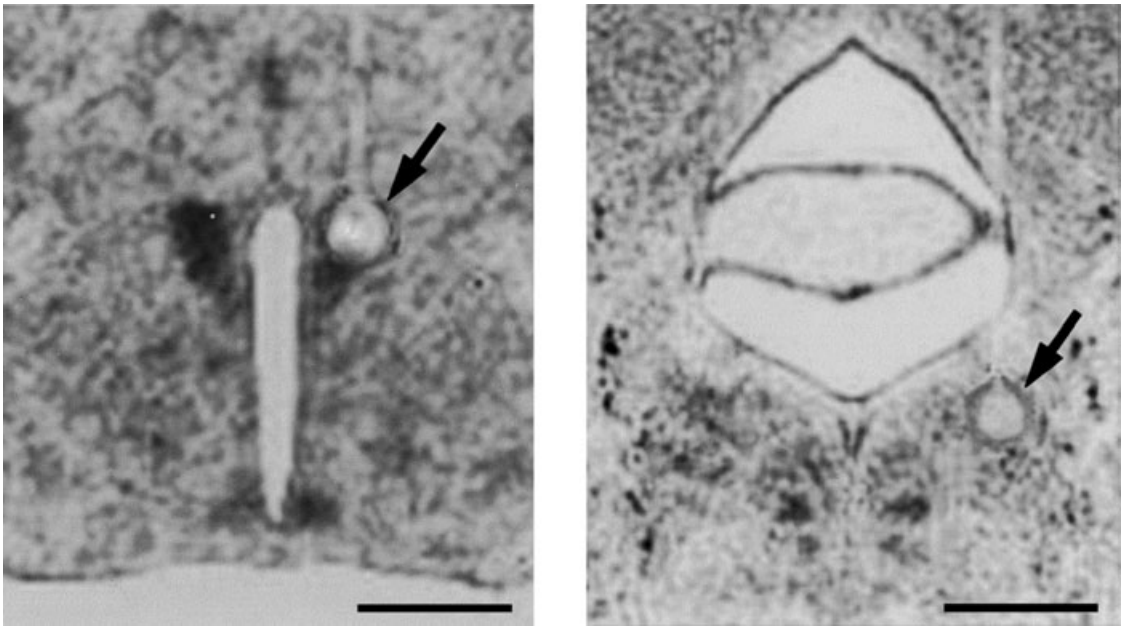

FIG. 1. Photomicrographs of transverse sections at the levels of the paraventricular nucleus (PVN) (A) and locus coeruleus (LC) (B) showing the electrolytic lesion in the right PVN and right LC (indicated by the arrows), and the intact nuclei on the corresponding left sites. Scale bars, 1 mm.

semi-microelectrode/micropipette pairs were clearly positioned within the PVN and LC were considered for posterior data analysis.

Systolic pressure

At 40 days of age rats were anesthetized with 1.2 g/kg urethane, placed in a stereotaxic apparatus, and two semi-microelectrode/micropipette pairs were advanced to the LC and PVN for microinjecting CRH into the LC and prazosin into the PVN, as previously described for the electrophysiological experiments. Systolic blood pressure was measured from the rat's tail by means of a non-invasive blood pressure system (XBP 1000, Kent Scientific Apparatus, Torrington, CT, USA) before and 2, 5, 10, 15, 20, 25 and 30 min after drug microinjection of CRH into the LC. The semi-microelectrodes of the two pairs were used for making electrolytic lesions (1-mA current for 20 s) to verify the correct placement of the semi-microelectrode/micropipette assemblies in the respective nuclei (see Fig. 1).

Data analyses

To determine the changes induced by the undernutrition regimen during pregnancy on the body weight of pregnant mothers, as well as on the body weight, brain weight and systolic blood pressure of pups, values obtained from undernourished animals were expressed as means ± SEM and compared with the corresponding values found in normal animals using an unpaired two-tailed Student's *t*-test.

In the electrophysiological experiments, a group of eight rats was initially used. In each of these animals multiunit neuronal activity was recorded from the LC and PVN and evaluated as follows: (i) counting of unambiguous action potentials directly from the computer screen (without including background noise) in three consecutive recording samples of 2 s each and (ii) computer-assisted measurement of the integrated multiunit activity (subtracting background noise) in the same three consecutive recording samples. Results from action potential counting and multiunit activity integration were then subjected to linear regression analysis (ORIGIN 6.0 software, Microcal Software, Inc., Northampton, MA, USA). Correlation coefficients were always higher than 0.95 ($P < 0.0001$) so integrated multiunit activity was used to evaluate drug effects on LC and PVN neurons throughout the electrophysiological study. Figure 2 illustrates the two semi-microelectrode/micropipette arrays positioned in the LC and PVN (Fig. 2A), a representative example of multiunit neuronal activity recorded from the LC and PVN of one rat before and 10 min after CRH microinjection into the LC (Fig. 2B), as well as the correlation between multiunit spike frequency and multiunit integrated activity performed in samples of neuronal recordings from the LC and PVN of eight rats before and 10 min after CRH microinjection into the LC (Fig. 2C). One-way ANOVA was used to analyse the time-course of effects of the drugs (intra-group comparisons) followed by the Dunnett multiple comparisons test (INSTAT 3.00, GraphPad Software Inc., San Diego, CA, USA). To appreciate the global effect of drugs or CSF over the total period of testing in both normal and undernourished animals, the area under the curves (AUC) was determined. The AUC was calculated as the integral from 0 to 30 min (period of testing) using the ORIGIN 6.0 software, and AUC change was defined as AUC under drug minus AUC under CSF and plotted as a bar graph. The effects of the drug treatment and the nutritional condition on AUC change (inter-group comparisons) were analysed using two-way ANOVA followed by the Bonferroni posthoc test (PRISM 3.00, GraphPad Software Inc.).

To assess the changes induced by microinjection of drugs into the LC and PVN on cardiovascular function, the systolic blood pressure was recorded. The time-course of drug effects (intra-group comparisons) was analysed using one-way ANOVA followed by the Dunnett multiple comparisons test. The global effect of drugs or CSF over the total period of testing of systolic pressure, in both normal and undernourished animals, was evaluated through the AUC change as described above, and the effect of drug treatments as well as the effect of the nutritional condition (inter-group comparisons) were analysed using two-way ANOVA followed by the Bonferroni posthoc test.

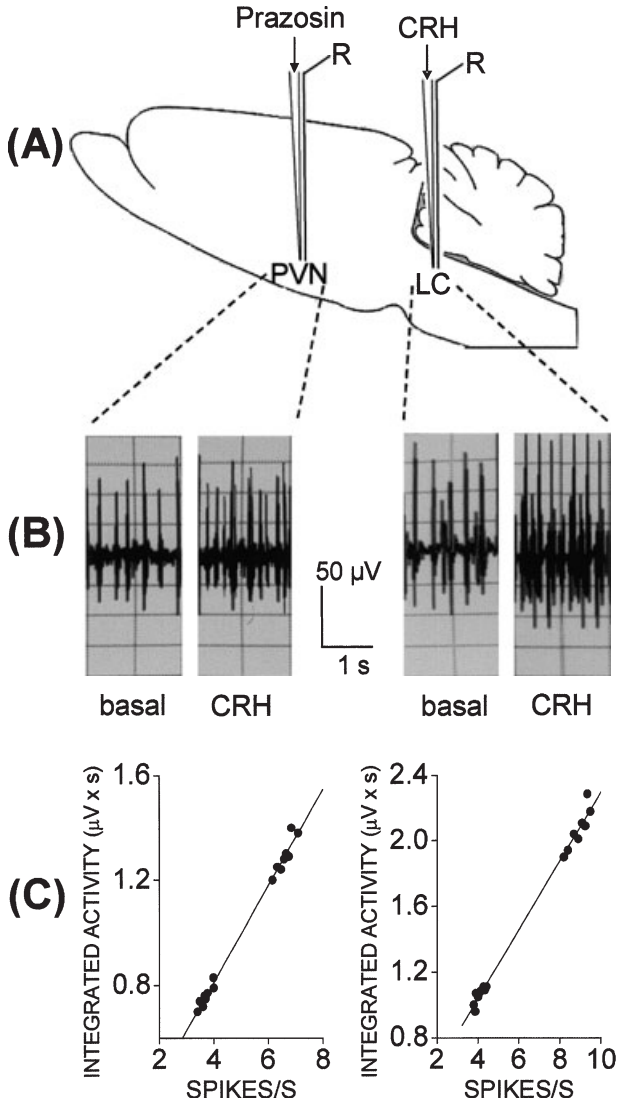

FIG. 2. (A) Scheme showing placement of the two semi-microelectrode/micropipette pairs advanced to the locus coeruleus (LC) [coordinates A, −1.6; L, 1.0; V, −2.8 (mm)] and paraventricular nucleus (PVN) [coordinates A, 6.4; L, 0.5; V, −1.6 (mm)]. R, recording semi-microelectrode. Corticotropin-releasing hormone (CRH) and prazosin were mechanically microinjected into the LC and PVN by means of the micropipettes associated with each microelectrode. (B) Representative examples of multiunit activity recording samples, of 2 s each, before (basal) and 10 min after microinjecting CRH (0.5 µg/0.05 µL) into the LC in one normal 40-day-old eutrophic rat; upward deflection potentials are negative. (C) Plot of multiunit integrated activity vs. multiunit spike frequency using samples of neuronal recordings from the LC and PVN of eight rats, before and 10 min after microinjection of CRH into the LC. Each of the eight points corresponds to the mean of three samples from one rat (three x/y pairs); the eight points that show lower integrated activity and spike frequency (left/down location in each graph) correspond to pre-CRH basal controls, and the eight points showing higher integrated activity and spike frequency (right/up location in each graph) correspond to post-CRH scores. Correlation coefficients of the regression lines were 0.978 ($P < 0.0001$) and 0.962 ($P < 0.0001$) for the right (LC) and left (PVN) graphs, respectively.

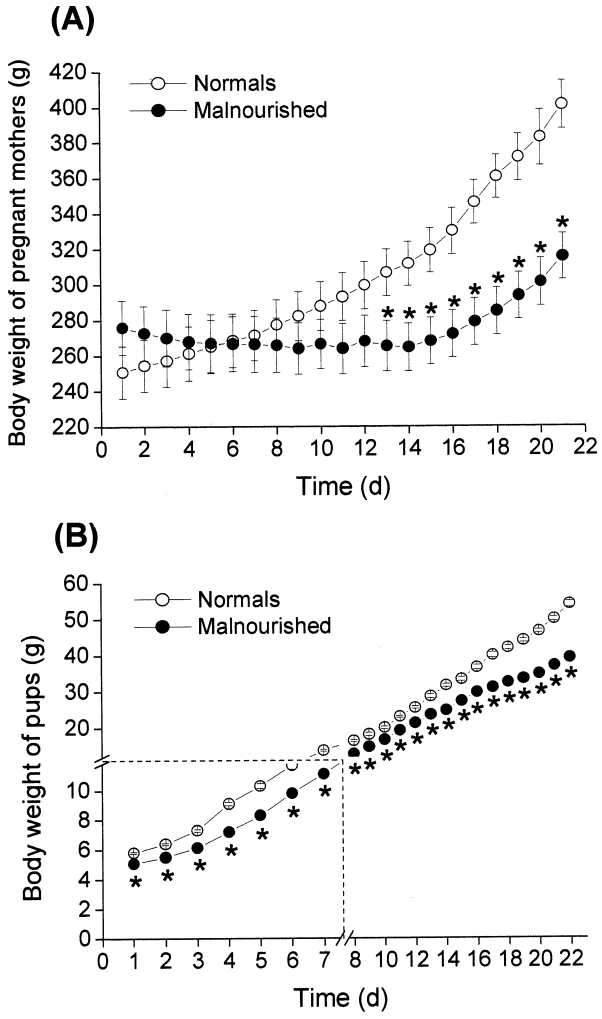

FIG. 3. (A) Body weight gain of pregnant rats submitted to *ad libitum* (normal group) or restricted (10 g/day) diet (undernourished group). Values are means ± SEM. *Significant differences ($P < 0.05$) in body weight between normal and undernourished rats (Student's *t*-test). (B) Body weight gain during the lactation period of normal and prenatally undernourished pups. Values are means ± SEM. *Significant differences ($P < 0.05$) in body weight between normal and undernourished groups (Student's *t*-test).

Results

Figure 3A shows that from day 8 to 20 of pregnancy, the mean body weight of undernourished dams was significantly lower than that of normal dams ($P < 0.05$). Figure 3B shows that undernourished pups exhibited, from day 2 of postnatal life until weaning, a significant body weight deficit compared with normal pups ($P < 0.05$). Table 1 shows that, at day 2 of postnatal life, undernourished animals had statistically lower body ($P < 0.001$) and brain ($P < 0.01$) weights than normal rats. Table 1 also shows that, at day 40 of postnatal life, body ($P < 0.01$) and brain ($P < 0.001$) weights of undernourished rats were significantly decreased, whereas a significant increase in systolic pressure ($P < 0.01$) was observed in these animals.

Multiunit neuronal activity in locus coeruleus and paraventricular nucleus: effects of microinjection of corticotropin-releasing hormone into the locus coeruleus and/or prazosin into the paraventricular nucleus

Figure 4A shows that at $t = 0$ min the basal integrated multiunit activity of LC neurons in the undernourished group was almost twice that of the normal group ($P < 0.01$). Microinjection of 0.5 µg CRH into the LC increased by about 100% the multiunit neuronal activity recorded from the LC of normal rats ($P < 0.05$ at 5, 10, 15 and 20 min

TABLE 1. Body weights and brain weights of normal and undernourished rats at days 2 and 40 of age

Parameter	Normal group	Undernourished group
Day 2		
Body weight (g)	6.7 ± 0.3 (10)	5.5 ± 0.2** (10)
Brain weight (mg)	228.5 ± 21.3 (10)	195.6 ± 18.6* (10)
Day 40		
Body weight (g)	153.6 ± 18.3 (32)	136.8 ± 15.1* (32)
Brain weight (mg)	1374.8 ± 27.9 (32)	1292.1 ± 30.5** (32)
Systolic pressure (mmHg)	129.3 ± 13.5 (16)	144.9 ± 15.4* (16)

Systolic pressure of normal and undernourished rats at day 40 of age. Values are means ± SD. Parentheses enclose number of rats. Significance of difference (Student's *t*-test) between normal and undernourished rats: *$P < 0.01$; **$P < 0.001$.

after CRH microinjection), whereas the LC activity of undernourished animals was not significantly modified. Figure 4 also shows that microinjection of 1.2 μg prazosin into the PVN induced a significant decrease of LC neuronal activity in the undernourished group ($P < 0.001$), the LC neurons of these animals reaching activity levels similar to those observed in the normal group. In contrast, the prazosin microinjection into the PVN did not modify the LC activity in normal rats but CRH administration within the LC 10 min after prazosin microinjection into the PVN induced significant increases of neuronal activity in the LC of both normal ($P < 0.05$, 5–20 min after CRH microinjection) and undernourished ($P < 0.05$, 10–30 min after CRH microinjection) rats. Thus, in the undernourished group, LC neuronal activity could only be enhanced by CRH after reducing the LC activity with prazosin. Figure 4B shows the global effect of the CRH microinjection over the total period of testing (30 min). It can be observed that CRH microinjection into the LC failed to activate LC neurons only in the undernourished animals without prazosin pretreatment ($P < 0.05$). In contrast, in prazosin-pretreated undernourished animals CRH microinjection into the LC produced neuronal activation, which means that administration of prazosin into the PVN modified the response of LC neurons to CRH in the undernourished group (significant interaction prazosin × nutritional condition in the two-way ANOVA, $P < 0.05$).

Figure 5A shows that at $t = 0$ min the basal integrated multiunit activity of PVN neurons in the undernourished group was at least twice that of the normal group ($P < 0.001$). Microinjection of 0.5 μg CRH into the LC increased by about 80% the multiunit neuronal activity recorded from the PVN of normal rats ($P < 0.05$ at 5, 10 and 15 min after CRH microinjection), whereas the PVN activity of undernourished animals was not significantly modified. Figure 5 also shows that microinjection of 1.2 μg prazosin into the

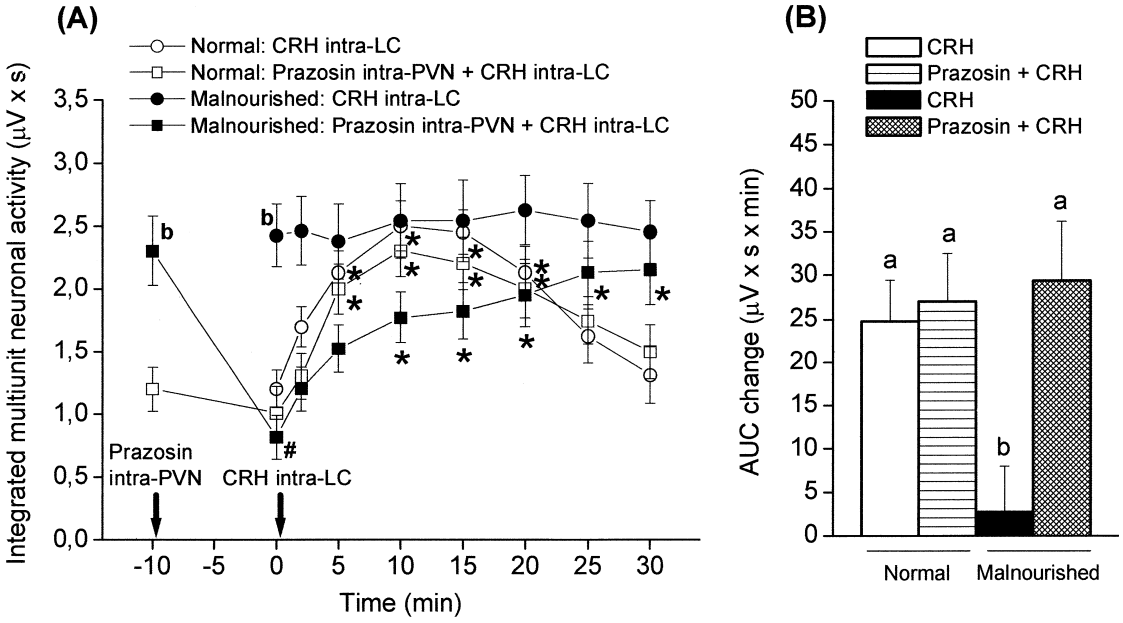

FIG. 4. (A) Time-course of integrated multiunit neuronal activity recorded from the locus coeruleus (LC) of normal and prenatally undernourished rats after intra-LC microinjection of corticotropin-releasing hormone (CRH) (0.5 μg/0.05 μL, right arrow) with or without prazosin pretreatment [intra-paraventricular nucleus (PVN) microinjection, 1.2 μg/0.05 μL, left arrow]. Values are means ± SEM, $n = 8$ rats in each group. The effect of the intra-LC microinjection of CRH over the time-course (intra-group comparison) was analysed using one-way ANOVA followed by the Dunnett posthoc test. Normal rats receiving only CRH microinjection, P ANOVA < 0.0001, $F = 6.676$; normal rats receiving CRH preceded by prazosin, P ANOVA < 0.0002, $F = 4.896$; undernourished rats receiving only CRH, P ANOVA $= 0.9991$, $F = 0.07912$; undernourished rats receiving CRH preceded by prazosin, P ANOVA < 0.0005, $F = 4.453$. In these series, the probability level for comparison of values obtained after CRH microinjection (at 2–30 min) with the corresponding value obtained before CRH microinjection (at 0 min) was *$P < 0.05$ (Dunnett multiple comparisons test). The effect of the prazosin microinjection into the PVN on multiunit neuronal activity recorded from the LC of normal and prenatally undernourished rats was analysed by comparing preprazosin values (at −10 min) with postprazosin values (at 0 min) using two-tailed Student's *t*-test, #$P < 0.001$. The effect of the nutritional condition was analysed by comparing corresponding values from the normal groups with that from the undernourished groups (at −10 and 0 min) using two-tailed Student's *t*-test, $^{b}P < 0.01$. (B) The area under the curves (AUC) was calculated as the integral from 0 to 30 min (ORIGIN 5.0 software, Microcal Software, Inc.); AUC change was defined as AUC under drug (data from A) minus AUC under cerebrospinal fluid (data from Fig. 6) and plotted as a bar graph. The effect of prazosin pretreatment as well as the effect of the nutritional condition on AUC change (intra-group comparisons) was analysed using two-way ANOVA followed by the Bonferroni posthoc test. Prazosin variable, P ANOVA < 0.05, $F = 3.03$; nutritional condition, P ANOVA < 0.05, $F = 6.60$; interaction for the two factors, P ANOVA < 0.05, $F = 4.71$. Inter-group comparisons (Bonferroni multiple comparisons test) indicated that means without common superscripts are significantly different ($P < 0.05$).

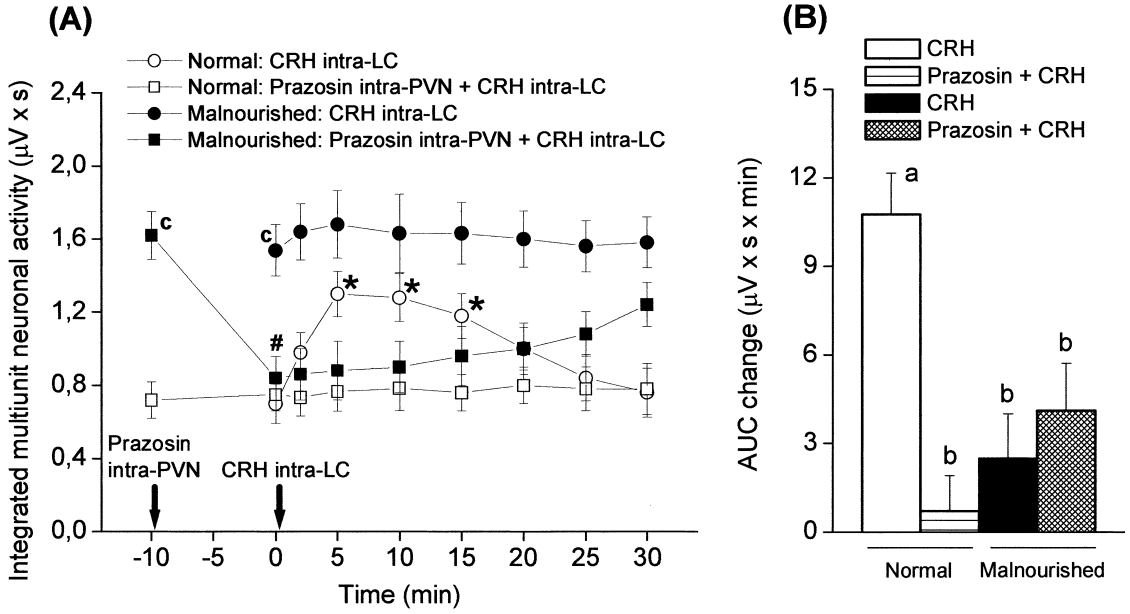

FIG. 5. (A) Time-course of integrated multiunit neuronal activity recorded from the paraventricular nucleus (PVN) of normal and prenatally undernourished rats after intra-locus coeruleus (LC) microinjection of corticotropin-releasing hormone (CRH) (0.5 μg/0.05 μL, right arrow) with or without prazosin pretreatment (intra-PVN microinjection, 1.2 μg/0.05 μL, left arrow). Values are means ± SEM, $n = 8$ rats in each group. The effect of the intra-LC microinjection of CRH over the time-course (intra-group comparison) was analysed using one-way ANOVA followed by the Dunnett posthoc test. Normal rats receiving only CRH microinjection, P ANOVA < 0.005, $F = 3.624$; normal rats receiving CRH preceded by prazosin, P ANOVA $= 0.2600$, $F = 1.316$; undernourished rats receiving only CRH, P ANOVA $= 0.9990$, $F = 0.0817$; undernourished rats receiving CRH preceded by prazosin, P ANOVA $= 0.4728$, $F = 0.9549$. In these series, the probability level for comparison of values obtained after CRH microinjection (at 2–30 min) with the corresponding value obtained before CRH microinjection (at 0 min) was $^{*}P < 0.05$ (Dunnett multiple comparisons test). The effect of the prazosin microinjection into the PVN on multiunit neuronal activity recorded from the LC of normal and prenatally undernourished rats was analysed by comparing preprazosin values (at −10 min) with postprazosin values (at 0 min) using two-tailed Student's t-test, $\#P < 0.001$. The effect of the nutritional condition was analysed by comparing corresponding values from the normal groups with that from the undernourished groups (at −10 and 0 min) using two-tailed Student's t-test, $^{c}P < 0.001$. (B) Area under the curves (AUC) changes and two-way ANOVA statistics were determined as in Fig. 4B. Prazosin variable, P ANOVA $= 0.1023$, $F = 2.85$; nutritional condition, P ANOVA < 0.01, $F = 8.68$; interaction for the two factors, P ANOVA < 0.0005, $F = 16.50$. Inter-group comparisons (Bonferroni multiple comparisons test) indicated that means without common superscripts are significantly different ($P < 0.001$).

PVN induced a significant decrease of PVN neuronal activity in the undernourished group ($P < 0.001$), the PVN neurons of these animals reaching activity levels similar to those observed in the normal group. In contrast, the prazosin microinjection into the PVN did not modify the PVN activity in normal rats but CRH administration into the LC (10 min after prazosin microinfusion within the PVN) failed to increase the neuronal activity in the LC of both normal and undernourished rats. Thus, in normal animals, LC neuronal activity could be enhanced by CRH only in the absence of prazosin. Figure 5B shows the global effect of the CRH microinjection over the total period of testing (30 min). It can be observed that CRH microinfusion into the LC failed to enhance LC neuronal activity in undernourished animals and in prazosin-pretreated normal rats. In contrast, in prazosin-free normal animals, CRH microinjection into the LC produced neuronal activation, which means that administration of prazosin into the PVN modified the response of LC neurons to CRH in normal animals (significant interaction prazosin × nutritional condition in the two-way ANOVA, $P < 0.0005$).

Figure 6 shows that the integrated multiunit activity of LC and PVN neurons in both normal and undernourished animals was not modified by microinjections of CSF into the PVN and LC. However, significant differences of neuronal activity can be observed when comparing normal and undernourished groups, the latter having about twice the integrated multiunit activity scores of the former throughout the recording period.

Systolic pressure: effects of microinjections of corticotropin-releasing hormone into the locus coeruleus and/or prazosin into the paraventricular nucleus

Figure 7A shows that at $t = 0$ min the systolic arterial pressure in the undernourished group was significantly higher than that in the normal group ($P < 0.01$). Microinjection of 0.5 μg CRH into the LC increased the systolic pressure recorded from normal rats ($P < 0.05$ at 5 and 10 min after CRH microinjection). Figure 7 also shows that microinjection of 1.2 μg prazosin into the PVN induced a significant decrease of the systolic pressure in the undernourished group ($P < 0.001$), which reaches scores similar to those observed in the normal group. In contrast, prazosin microinjected into the PVN did not modify the systolic pressure in normal rats. When CRH was administered within the LC, 10 min after prazosin microinjection into the PVN, it induced significant increases of systolic pressure of both normal ($P < 0.05$, 10 min after CRH microinjection) and undernourished ($P < 0.05$, 25 and 30 min after CRH microinjection) rats. Thus, in undernourished animals, the systolic pressure could only be increased by CRH after reducing the arterial blood pressure with prazosin. Figure 7B shows the global effect of the CRH microinjection over the total period of testing (30 min). It can be observed that CRH microinjection into the LC failed to induce systolic pressure increases only in undernourished animals without prazosin pretreatment ($P < 0.05$). In contrast, in prazosin-pretreated undernourished animals, CRH into the LC produced a systolic pressure enhancement,

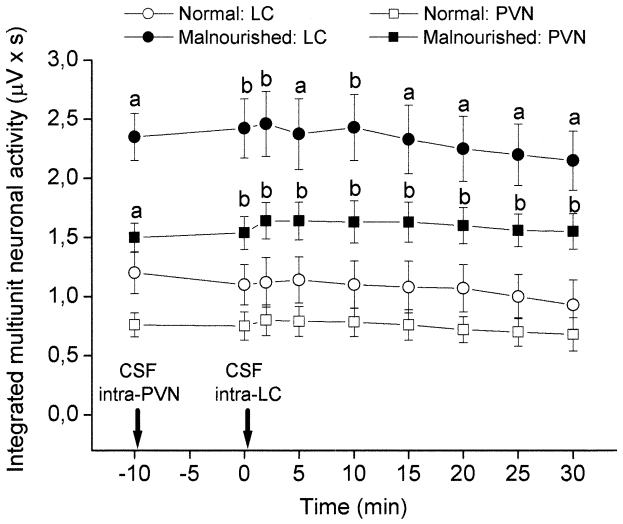

FIG. 6. Multiunit neuronal activity recorded from the locus coeruleus (LC) and paraventricular nucleus (PVN) of normal and prenatally undernourished rats after microinjection of 0.05 μL of cerebrospinal fluid (CSF) intra-PVN (left arrow) and intra-LC (right arrow). Values are means ± SEM, $n = 8$ rats in each group. The effect of CSF microinjection into the PVN and LC over the time-course (intra-group variable) as well as the effect of the nutritional condition (inter-group variable) on the multiunit neuronal activity were analysed using two-way ANOVA followed by the Bonferroni posthoc test. CSF microinjection into both nuclei induced no significant changes in multiunit neuronal activity recorded from the LC and PVN of normal and prenatally undernourished rats (PVN, P ANOVA $= 0.9936$, $F = 0.10$; LC, P ANOVA $= 0.9704$, $F = 0.28$), whereas the nutritional condition significantly affected the multiunit neuronal activity recorded from both nuclei (PVN, P ANOVA < 0.0001, $F = 165.64$; LC, P ANOVA < 0.0001, $F = 127.92$); interaction for the two factors, P ANOVA $= 0.9999$, $F = 0.05$. Inter-group comparisons (Bonferroni multiple comparisons test) indicated that undernourished groups had significantly higher scores of neuronal activity than normal groups ($^{a}P < 0.01$, $^{b}P < 0.001$).

indicating that preinfusion of prazosin into the PVN modified the effect of CRH on systolic blood pressure of undernourished animals (significant interaction prazosin × nutritional condition in the two-way ANOVA, $P < 0.05$). It can then be observed that the effect on systolic pressure produced by intra-LC CRH in both normal and undernourished rats closely parallels that elicited by the peptide in multiunit neuronal activity of the LC (see Fig. 4).

Discussion

The reduction of food intake during pregnancy resulted in a lower maternal weight gain as well as in a significant body weight deficit of the offspring at birth, which is indicative of fetal growth retardation. Brain weight measurements performed in rats of 2 and 40 days of age revealed a significant brain weight deficit in the undernourished group. As reported previously, prenatal malnutrition could result in long-lasting brain weight deficits through a mechanism involving losses of neurons, glia and myelin, and impaired dendritic differentiation, among other factors (Morgane *et al.*, 1993).

At 40 days of age, a significant increase of systolic pressure was observed in undernourished rats. As mentioned above, hypertension can develop in rat progeny by submitting normotensive rat mothers to undernutrition during pregnancy (Langley-Evans *et al.*, 1996a,b; Zicha & Kunes, 1999; Perez *et al.*, 2002), a hypertensive state that is mainly due to increase in systolic pressure. As discussed elsewhere (for review see Seckl, 2001), hypertension in the offspring is probably caused by a series of sequential events induced by prenatal malnutrition including: (i) decreased activity of placental 11β-hydroxysteroid dehydrogenase type 2 which catalyses the rapid metabolism of cortisol and corticosterone to inert steroids, resulting in increased exposure of the fetal brain to glucocorticoids of maternal origin (Langley-Evans *et al.*, 1996a,b); (ii) this leads to decreased glucocorticoid receptor expression during fetal life in regions concerned with the regulation of the hypothalamus-pituitary-adrenal axis, such as the hypothalamus (Bertram *et al.*, 2001), pituitary (Hawkins *et al.*, 2001) and hippocampus (Lesage *et al.*, 2001), a structure with the highest density of corticosteroid binding sites in the brain and an important site of feed-back control upon the hypothalamus-pituitary-adrenal axis (Levitt *et al.*, 1996; Welberg *et al.*, 2001); (iii) the reduced negative feed-back control by glucocorticoids results in higher expression of CRH during postnatal development (Perez *et al.*, 2004), a peptide serving as a positive signal to the hypothalamus-pituitary-adrenal axis (causing increased plasma levels of corticosterone) but also to extra-hypothalamic brain regions such as the LC (Dunn *et al.*, 2004); and (iv) excitation of both PVN and LC neurons may account for the hypertensive state observed in prenatally undernourished rats, as PVN (Palkovits, 1999) and LC (Drolet & Gauthier, 1985, 1987) neurons may activate the sympathoadrenomedullary system. In addition, activation of LC neurons could lead to hypertension by depressing the baroreceptor reflex tone (Chan *et al.*, 1992).

The foregoing results indicate that intra-coerulear microinjection of CRH in normal rats markedly increased the multiunit neuronal activity in the LC. Although it has been universally observed that intracerebroventricular administration of CRH activates LC-noradrenergic neurons electrophysiologically, there exists some controversy concerning the source and precise target of CRH. Valentino *et al.* (1983) argued that the action of CRH is exerted directly on LC-noradrenergic neurons. This evidence is consistent with anatomic studies that have indicated nerve terminals positive for CRH immunoreactivity in apparent contact with neurons containing tyrosine hydroxylase immunoreactivity and thus presumed to be noradrenergic (Valentino *et al.*, 1992; Van Bockstaele *et al.*, 1998). However, not all CRH-containing terminals existing in the LC come from the PVN, and other potential sources include the nucleus paragigantocellularis (Valentino *et al.*, 1992) and the central nucleus of the amygdala (Van Bockstaele *et al.*, 1998). Other studies have suggested that the effect of CRH on LC-noradrenergic neurons is indirect, on the basis that the release of norepinephrine in the hippocampus in response to CRH locally applied into the LC occurred with an average delay of 6–7 min (Dunn *et al.*, 2004). More recently, however, unequivocal evidence has been provided that some neurons in the PVN have monosynaptic associations with catecholaminergic dendrites in the LC (Reyes *et al.*, 2005). Moreover, *in vitro* studies have shown that CRH directly activates noradrenergic neurons of the LC (Jedema & Grace, 2004).

The present results also show that, in addition to the increased neuronal activity in the LC, intra-coerulear microinjection of CRH in normal rats also enhanced the multiunit neuronal activity in the PVN. Activation of PVN neurons by intra-coerulear CRH was probably the result of activation of feed-forward LC–PVN excitatory noradrenergic connections, as microinfusion of the α_1 antagonist prazosin into the PVN before intra-coerulear CRH injection prevented the observed excitatory effect in PVN neurons. Although other noradrenergic nuclei also provide an important noradrenergic input to the PVN, direct projections from the LC to the PVN have already been reported (Cunningham & Sawchenko, 1988; Saphier, 1989, 1993; Petrov *et al.*, 1993; Hwang *et al.*, 1998). In addition, the existence of such a closed positive feed-forward loop reciprocally interconnecting the PVN and LC via CRHergic and noradrenergic projections has been suggested previously (Dunn & Berridge, 1990; Dunn *et al.*, 2004).

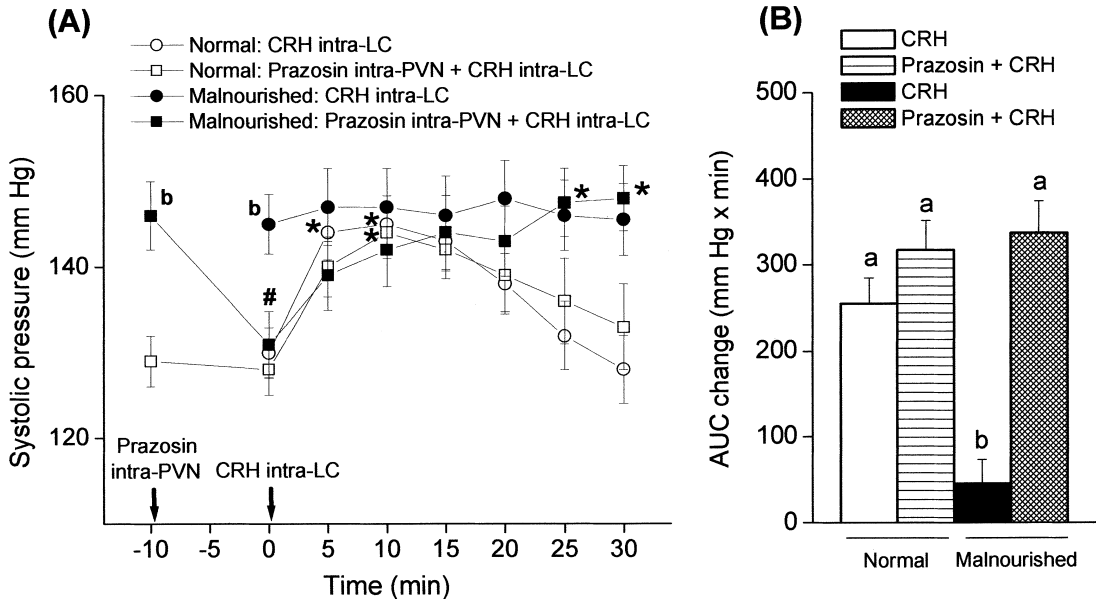

FIG. 7. (A) Time-course of systolic arterial pressure of normal and prenatally undernourished rats after intra-locus coeruleus (LC) microinjection of corticotropin-releasing hormone (CRH) (0.5 μg/0.05 μL, right arrow) with or without prazosin pretreatment [intra-paraventricular nucleus (PVN) microinjection, 1.2 μg/0.05 μL, left arrow]. Values are means ± SEM, $n = 8$ rats in each group. The effect of the intra-LC microinjection of CRH over the time-course (intra-group comparison) was analysed using one-way ANOVA followed by the Dunnett posthoc test. Normal rats receiving only CRH microinjection, P ANOVA < 0.0001, $F = 6.676$; normal rats receiving CRH preceded by prazosin, P ANOVA < 0.0002, $F = 4.896$; undernourished rats receiving only CRH, P ANOVA $= 0.9991$, $F = 0.0791$; undernourished rats receiving CRH preceded by prazosin, P ANOVA < 0.0005, $F = 4.453$. In these series, the probability level for comparison of systolic pressures obtained after CRH microinjection (at 2–30 min) with the corresponding systolic pressure measured before CRH microinjection (at 0 min) was $^{*}P < 0.05$ (Dunnett multiple comparisons test). The effect of the prazosin microinjection into the PVN on systolic pressure of normal and prenatally undernourished rats was analysed by comparing preprazosin values (at −10 min) with postprazosin values (at 0 min) using two-tailed Student's t-test, $\#P < 0.001$. The effect of the nutritional condition was analysed by comparing corresponding systolic pressures from the normal groups with that from the undernourished groups (at −10 and 0 min) using two-tailed Student's t-test, $^{b}P < 0.01$. (B) Area under the curves (AUC) changes and two-way ANOVA statistics were determined as in Fig. 4B (data of the effect of cerebrospinal fluid microinjection into the PVN and LC on systolic pressure are not shown). Prazosin variable, P ANOVA < 0.05, $F = 3.03$; nutritional condition, P ANOVA < 0.05, $F = 6.60$; interaction for the two factors, P ANOVA < 0.05, $F = 4.71$. Inter-group comparisons (Bonferroni multiple comparisons test) indicated that means without common superscripts are significantly different ($P < 0.05$).

In contrast to normal rats, in undernourished rats the intra-coerulear microinjection of CRH failed to enhance neuronal activity in the LC. Interestingly, previous microinfusion of prazosin into the PVN restored the ability of CRH to excite LC neuronal activity in these animals. At least two alternative possibilities may account for this observation: (i) CRH receptors in LC neurons were desensitized because of CRH hyperactivity presenting prenatally undernourished rats; and/or (ii) LC neurons are fully active in prenatally undernourished animals and therefore insensitive to further excitation by exogenous CRH. The former alternative is supported by the fact that increased CRH mRNA expression has been reported in the hypothalami of rats undernourished *in utero* (Perez *et al.*, 2004), although increased release of CRH in prenatal undernutrition-induced hypertensive states has not been reported so far. In addition, CRH receptors in the brain can undergo down-regulation, as has been found in the cerebral cortex of rats after chronic exposure to stress (Anderson *et al.*, 1993; Iredale *et al.*, 1996) and in a model of *in vitro* human neuroblastoma cells after chronic administration of CRH (Roseboom *et al.*, 2001). Nevertheless, the fact that prazosin locally applied into the PVN restored the capacity of CRH to activate LC neurons in undernourished animals argues against CRH receptor desensitization as a possible factor involved in LC irresponsiveness to CRH administration, pointing rather to the second alternative as a more plausible mechanistic explanation. In fact, in undernourished rats the LC and PVN basal neuronal activity (without drugs) was about twice that of normal rats, making it less possible that LC neurons are excited by exogenous CRH. In this regard, data from the literature show that the spontaneous discharge rate of LC neurons cannot be enhanced by more than two-fold after high doses of intra-LC (Curtis *et al.*, 1997) and i.c.v. (Conti *et al.*, 1997; Curtis *et al.*, 1997) CRH. Increased activity of LC neurons (60–70% increase in a single-unit recording paradigm) has been previously described in rats submitted to a perinatal undernutrition regimen (Nasif *et al.*, 2001). Thus, it seems likely that LC neurons in undernourished animals are fully active in a range that mimics the effect of a high CRH dose (i.e. 10 μg i.c.v.). In agreement with this interpretation, the intra-PVN microinjection of prazosin in undernourished rats decreased LC neuronal activity to levels found in normal animals, thus allowing LC cells to regain full responsiveness to the CRH challenge. This effect of prazosin in undernourished rats is likely to be mediated by α_1-adrenoceptor blockade at the PVN level, thereby disrupting the feed-forward loop and resulting in diminished activity of both PVN and LC interconnected neurons. As noted by Dunn *et al.* (2004), in some conditions (stress, panic) reciprocal activation between LC and PVN neurons could potentially lead to a vicious cycle with the mutual activation escalating the activity of noradrenergic and CRHergic systems in an uncontrolled manner. This could also be occurring in neurons of both the LC and PVN of prenatally undernourished rats, on the basis of the enhanced brain noradrenergic activity (Soto-Moyano *et al.*, 1998a,b) and the increased hypothalamic CRH mRNA expression (Pérez *et al.*, 2004) observed in these animals.

Microinjection of CRH into the LC of normal rats produced slight but significant elevations of systolic pressure, which is in agreement with previous reports showing increased arterial blood pressure after

i.c.v. (Brown *et al.*, 1988) and intra-LC (Ku *et al.*, 1998) administration of CRH. Intra-cerebral CRH seems to increase blood pressure by activating the sympathetic-adrenomedullary system as, on the one hand, centrally administered CRH resulted in both activation of adrenal sympathetic efferent nerve activity (Kurosawa *et al.*, 1986) and increased plasma norepinephrine and epinephrine (Nijsen *et al.*, 2000) and, on the other hand, sympathetic blockade prevented CRH-induced tachycardia (Nijsen *et al.*, 2000). The fact that prazosin microinjected into the PVN 10 min before CRH microinfusion into the LC of normal animals did not prevent the increases in blood pressure suggests that the LC to PVN connection seems to be non-essential for eliciting the cardiovascular effect observed in response to exogenously administered CRH. In other words, it seems likely that in the present experiments the sympathetic-adrenomedullary system was stimulated via LC activation but not via PVN activation. In the brain, CRH can stimulate the G_s-adenylyl cyclase system (Grammatopoulos & Chrousos, 2002), a pathway that has been reported to induce neuronal excitation in the LC (Nestler *et al.*, 1999). Some studies have reported CRH to be ineffective in modifying blood pressure after intracerebroventricular (Schulz *et al.*, 1994) and intra-coerulear (Curtis *et al.*, 1997) administration in the rat. In this respect, it has been argued that centrally administered CRH is unable to increase blood pressure in anesthetized rats (Schulz *et al.*, 1994). Nevertheless, recent data showed that LC neurons are similarly activated by CRH in anesthetized and unanesthetized rats, as revealed by the chronoamperometric measurement of norepinephrine release in target brain sites for LC innervation (Dunn *et al.*, 2004). In the present study, CRH administered into the LC of urethane-anesthetized rats gave rise to a slight but significant enhancement of systolic pressure, similar to that reported by Ku *et al.* (1998).

In contrast to the effects induced in normal rats, microinjection of CRH into the LC of hypertensive prenatally undernourished rats did not produce significant changes in systolic pressure. Similar results have previously been found by others in spontaneously hypertensive rats receiving i.c.v. CRH (Brown *et al.*, 1988). The ineffectiveness of intra-LC CRH in increasing systolic pressure in hypertensive prenatally undernourished rats cannot be a consequence of the down-regulation of CRH receptors in the brain as intra-PVN prazosin decreased the systolic pressure and concomitantly restored the ability of CRH to produce cardiovascular stimulation. It seems apparent therefore that the excitatory effects of CRH on cardiovascular activity could be exerted only in normotensive animals whereas, in malnutrition-induced hypertension, intra-LC CRH was unable to elicit cardiovascular effects beyond the limits imposed by an already hyperactive system.

The foregoing electrophysiological and cardiovascular results seem to support the existence of an excitatory closed loop reciprocally interconnecting PVN and LC neurons, which would be hyperactive during the postnatal life of animals submitted to maternal undernutrition and thereby responsible for the hypertensive state observed in these animals. In fact: (i) CRH receptor activation in the LC was capable of stimulating the activity of neurons in both the LC and PVN of well-nourished rats, whereas antagonizing α_1-adrenoceptors in the PVN with prazosin was able to disrupt the closed loop in undernourished animals; (ii) CRH failed to stimulate the feed-forward loop in prenatally undernourished hypertensive rats, a loop probably already activated in these animals and therefore insensitive to further excitation by the infused agonist; in contrast, the loop was highly sensitive to prazosin blockade in undernourished rats, a behavior which is expected to occur in an already activated neural network; and (iii) pharmacological stimulation of CRH receptors in the LC and α_1-adrenoceptor blockade in the PVN gave rise to a cardiovascular change largely agreeing with the electrophysiological data, i.e. normal animals were sensitive to CRH (as microinjection of the peptide increased systolic pressure), whereas undernourished animals were insensitive to CRH but sensitive to prazosin (as microinjection of the α_1 blocker decreased systolic pressure).

It seems worthwhile to point out that increases in hypothalamic CRH mRNA expression (Pérez *et al.*, 2004) and in central noradrenergic activity (Soto-Moyano *et al.*, 1998a) reported in prenatally malnourished rats are coherent with the increased tonic activity in the PVN–LC feed-forward loop proposed herein and by others (Dunn & Berridge, 1990; Dunn *et al.*, 2004), giving molecular support to such a notion. In this respect, an altered number of α- and β-adrenoceptors has already been observed in the total brain (Keller *et al.*, 1982) and neocortex (Seidler *et al.*, 1990; Soto-Moyano *et al.*, 2005) of perinatally undernourished rats, together with increased norepinephrine turnover (Marichich *et al.*, 1979) and release (Soto-Moyano *et al.*, 1998a,b). Further studies on adaptive changes occurring in brainstem adrenergic and hypothalamic CRHergic systems would be helpful for a better understanding of the hypothalamic–coerulear mechanisms by which maternal undernutrition leads to fetal programming of postnatal hypertension. Finally, the present data shed new light on the origin of prenatal undernutrition-induced hypertension in the rat, which is complementary to other hypotheses involving reduced nephron number in the kidney (Dodic *et al.*, 2002) and increased vascular glucocorticoid receptor number (Langley-Evans *et al.*, 1996c; Yang & Zhang, 2004), as part of the mechanisms programming hypertension in adult life.

Acknowledgements

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Abbreviations

AUC, area under the curves; CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; LC, locus coeruleus; PVN, paraventricular nucleus.

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