EXPERIMENTAL STUDY

Effects of corticotropin-releasing hormone on locus coeruleus neurons in vivo: a microdialysis study using a novel bilateral approach

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Abstract
Objective: Stress-induced release of noradrenaline (NA) from locus coeruleus (LC) neurons is mainly regulated by corticotropin-releasing hormone (CRH). Tyrosine is a precursor of NA and plays an intriguing role in the regulation of NA release.
Design: We studied the effects of injecting CRH into the LC using a novel bilateral approach which relies on the mainly ipsilateral projections of LC neurons allowing stimulation of one hemisphere while using the other as control. To analyze the modification of the CRH effect, tyrosine was given intraperitoneally. A combination of CRH and its antagonist D-Phe was administered for validation of the specificity of CRH effects.
Methods: Wistar rats were used in all experiments. Injections were made through fused silica capillaries implanted into both LCs and microdialysis samples were collected bilaterally from the prefrontal cortex (PFM) every 20 min for 1 h before and 3 h after injections. The effects of LC stimulation were investigated by determining 3-methoxy-4-hydroxyphenylglycol (MHPG) in the dialysates.
Results: Following CRH injection into one LC and contralateral infusion of artificial cerebrospinal fluid (aCSF), MHPG levels, which are indicative of NA release, increased only in the ipsilateral PFM. These effects were blocked by D-Phe. Simultaneous administration of tyrosine i.p. led to a significant prolongation of MHPG release.
Conclusions: These data provide the first physiological evidence of unilateral LC projections with the bilateral stimulation design proving to be a very valuable tool for the study of LC firing rate, to decrease number of animals and time expenditure. Prolongation of MHPG release after tyrosine supplementation is most likely due to increased NA synthesis.

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Introduction
Since the description of the 41-amino acid neuropeptide corticotropin-releasing hormone (CRH) by Vale et al. in 1981 (1), great interest has been focused on CRH not only as a stimulus for ACTH secretion from the anterior pituitary gland, but also as a central regulator of the stress response (2–4), a mediator of anxiety (5), for its role in appetite control (6, 7) and for its involvement in memory formation (8).

Consistent with the variety of actions mediated by CRH, this neuropeptide and its receptors have been found to be widely distributed over the whole central nervous system (CNS) (9, 10), including the locus coeruleus (LC) (11). This brainstem nucleus contains the largest number of noradrenergic cell bodies within the CNS and plays a dominant role in the integration of stress. Evidence has been provided that CRH-containing neurons synapse onto catecholaminergic dendrites within the LC (12) and numerous studies have shown that CRH enhances LC neuronal activity (see 13 and 14 for review). Direct injection of CRH into the LC elevates levels of noradrenaline (NA) and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in the parietal cortex, an effect which is attenuated by coinfusion of the CRH receptor antagonist α-helical CRH (15). Similar results were obtained in the prefrontal cortex (PFM) (16), which also receives dense noradrenergic innervation from the LC (17). Neuroanatomical data clearly demonstrate that LC fibers almost exclusively project to the ipsilateral cortex, with only a small percentage of projections into the contralateral hemisphere (18, 19). Based on these observations our objective was to obtain physiological evidence for this neuroanatomical finding and to develop a novel injection design that allows comparison of the LC stimulation between the two hemispheres.
We also aimed to study the effects of intraperitoneal (i.p.) application of the catecholamine precursor amino acid tyrosine. Under resting conditions, tyrosine hydroxylase (TH), the rate-limiting enzyme in NA synthesis, is nearly saturated, thus physiological levels of tyrosine will not affect catecholamine synthesis (20). However, under conditions of neuronal depolarization, the phosphorylation of TH leads to conformational changes in the enzyme that makes it dependent upon the availability of its substrate tyrosine (21). Under these conditions, tyrosine can in fact increase NA availability of its substrate tyrosine (21). Under these conditions, tyrosine can in fact increase NA synthesis (22, 23). To prove the specificity of stimulation, a third group of animals was injected with both CRH and its receptor antagonist d-Phe(12–41), which has been shown to be more potent than α-helical CRH (24) and does not possess intrinsic activity (25).

Materials and methods

Male Wistar rats (280–350 g, obtained from Harlan-Winkelmann, Borden, Germany) were used in all experiments. They were housed in small groups (3–4 animals) under a 12/12 h light/dark cycle with access to food and water ad libitum. Surgery was carried out 1 day prior to experiment under ketamine (Ketanest, Parke-Davis GmbH, Freiburg, Germany, 80 mg/kg i.p.) and 2% xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany, 12 mg/kg i.m.) anesthesia. Home-made concentric microdialysis probes (3 mm active length, membrane with a molecular cut-off at M, 50 000 (AN69HF, Hospal Nuernberg, Germany)) were implanted bilaterally into the PPM of the animals (+3.2 mm anterior and ±3.0 mm lateral to bregma, with the tip 3.2 mm below the brain surface according to the atlas of Paxinos and Watson (26)). Microinjection units made from fused silica capillary (150 μm o.d.) were implanted bilaterally into the LC (−1.3 mm posterior and ±1.4 mm lateral to lambda, with the tip 6.4 mm below the brain surface). After surgery, the animals were housed individually in special cages for recovery. Perfusion of the microdialysis probes was started using artificial cerebrospinal fluid (aCSF) according to Sharp & Zetterström (27), with the flow rate set at 0.5 μl/min.

On the following day, the flow rate was increased to 2.0 μl/min and tubings prefilled with the substances for application were attached to the microinjection units. An equilibration time of 2 h was allowed. Subsequently, the experiment was started on freely moving animals and 40 μl samples were collected on ice every 20 min in 10 μl 0.5 M perchloric acid. After the third fraction, i.e. after 60 min, injections were performed. The following groups were studied. Group 1 (n = 8): injection of 100 ng CRH in 0.5 μl aCSF into one LC, 0.5 μl aCSF into the contralateral LC and 0.5 ml NaCl i.p.; Group 2 (n = 8): injections of 100 ng CRH mixed with 500 ng d-Phe(12–41) in 0.5 μl aCSF into one LC, 0.5 μl aCSF into the contralateral LC and 0.5 ml NaCl i.p.; Group 3 (n = 8): injections of 100 ng CRH in 0.5 μl aCSF into one LC, 0.5 μl aCSF into the contralateral LC and 200 mg/kg tyrosine methyl ester i.p.

Injection time into the LC was 1 min. After injection, nine more samples were collected. All samples were stored at −80°C for later analysis. The samples were analyzed for the NA metabolite MHPG using reversed phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The system consisted of a Bischoff HPLC compact pump (Leonberg, Germany, pump rate 0.5 ml/min), a Rheodyne 7125 injection valve (Cotati, CA, USA), a 125 mm 3 μm Spherisorb 3 ODS 2 column (CS, Langerwehe, Germany) and a Coulochem II detector (ESA, Bedford, MA, USA) with an ESA 5011 analytical cell. Potentials were set to E1 = −50 mV for conditioning and E2 = +380 mV for oxidation, the mobile phase contained 100 mM NaH2PO4, 100 μM decansulfonic acid, 100 μM EDTA and 4% methanol adjusted to pH 4.20.

At the end of the experiment, the animal was given a lethal dose of pentobarbital (Narcoren, Rhone-Merieux GmbH, Laupheim, Germany, 300 mg/kg i.p.) and 0.5 μl Evans Blue was injected through the fused silica capillary for validation of the correct placement of the injection units. Brains were removed and stored in 4% phosphate-buffered formaldehyde solution. Only those animals with the injection cannula correctly placed were included in the statistical analysis. The number of animals per group (n = 8) mentioned above refers to those animals validated with correct placement in the LC.

Analysis of variance (ANOVA) was performed on raw data to determine statistical changes over time. Identification of statistical significance was provided by subsequent performance of Student’s paired t-test. For better comparison between groups, the data in Figs 1–3 are normalized: the mean value before stimulation was set at 100% and the presented columns show the relative change in percent.

Results

After stimulation of one LC with CRH and infusion of aCSF into the contralateral side, we found a highly significant (P < 0.01) maximal increase in sample 7 of the stimulated hemisphere of up to 138% when compared with levels before stimulation. These changes were still significant in samples 8 and 9 in the stimulated hemisphere with 132 and 120%, respectively (P < 0.05). No corresponding changes could be observed in the hemisphere treated with aCSF (Fig. 1).

Coinfusion of d-Phe (12–41) with CRH into one LC prevented elevation of MHPG levels (Fig. 2).

After i.p. administration of tyrosine, significant increases (P < 0.05 and P < 0.01) could be detected in samples 8–12, i.e. until cessation of sample
MHPG levels in these samples were elevated up to 136% in sample 7, 128% in sample 8, 136% in sample 9, 135% in sample 10, 144% in sample 11 and 136% in sample 12 when compared with levels before stimulation. Again, no changes could be observed on the control side treated with aCSF (Fig. 3). The dose of tyrosine used has been previously shown by us to sufficiently raise brain tyrosine levels (22).

**Figure 1** Relative change in MHPG levels compared with baseline after stimulation of one LC with CRH (filled columns) and injection of aCSF into the contralateral LC (grey columns). Columns and error bars represent means and S.E.M. (*P < 0.05 and **P < 0.01 when compared with baseline; + P < 0.05 when compared with contralateral hemisphere, n = 8).

**Figure 2** Relative change of MHPG levels compared with baseline after coinfusion of d-Phe(12–41) and CRH into one LC (filled columns) and infusion of aCSF into the contralateral LC (grey columns) (means ± S.E.M., n = 8).
Discussion

After administration of CRH, MHPG in the extracellular space was significantly elevated compared with baseline levels, indicating an increased firing rate of noradrenergic LC neurons. Together with the finding that the effect of CRH can be blocked by the coinfusion of the CRH receptor antagonist d-Phe (12–41), this clearly demonstrates that the effects of CRH on the LC are specific and mediated via the CRH receptor. These data confirm the results of previous studies, that have examined the CRH actions by various experimental techniques (15, 28, 29).

More importantly, while the unilateral injection of CRH did increase the firing rate of LC neurons, the contralateral hemisphere following aCSF injection was completely unaffected, proving in an in vivo experiment the neuroanatomical finding that the LC projections to the cortex are almost exclusively ipsilateral (18, 19). This study is, to our knowledge, the first to use the described bilateral design which offers several advantages. The interindividual variability that had to be taken into account in previous studies, that have examined the CRH actions by various experimental techniques (15, 28, 29).

A comparison of Groups 1 and 3 demonstrates that the application of tyrosine leads to a prolonged increase of MHPG in the PFM. In animals receiving saline i.p. as a control (Group 1), elevated MHPG levels on the stimulated hemisphere had returned to basal levels by sample 10, whereas MHPG remained elevated in tyrosine-treated animals (Group 3) until cessation of the experiment (sample 12). Although it cannot be excluded that the administration of tyrosine leads to a reduced rate of MHPG metabolism or changes in transport rates, thus preventing neurotransmitter levels from returning to basal levels, it is more likely that the synthesis of NA is enhanced following tyrosine application thus leading to a prolonged elevation of extracellular MHPG. There is evidence for this hypothesis from a number of studies investigating the allosteric and transcriptional regulation of TH, the rate-limiting enzyme in NA synthesis (21, 30).

Our results also shed new light upon the time course of CRH action and suggest that termination of CRH-induced noradrenergic activation is not due to cessation of the CRH effect at its receptor only, but also due to a decreased NA synthesis caused by tyrosine depletion.

References


