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DIFFERENTIAL EFFECTS OF CRH INFUSION INTO THE CENTRAL NUCLEUS OF THE AMYGDALA IN THE ROMAN HIGH-AVOIDANCE AND LOW-AVOIDANCE RATS

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SUMMARY

Roman-high (RHA/Verh) and low (RLA/Verh) avoidance rats are selected and bred for rapid learning versus non-acquisition of two-way, active avoidance behaviour in a shuttle box. RHA/Verh rats generally show a more active coping style than do their RLA/Verh counterparts when exposed to various environmental challenges. The central nucleus of the amygdala (CeA) is known to be involved in the regulation of autonomic, neuroendocrine and behavioural responses to stress and stress-free conditions, and it is considered in relation to coping strategies. Corticotropin-releasing hormone (CRH) seems to be a key factor in the control of the CeA output. Neuroanatomical studies have revealed that the majority of CRH fibers from the CeA have direct connections with autonomic regulatory nuclei in the brainstem, e.g. lateral parabrachial nucleus (lPB), ventrolateral periaquaductal gray (vlPAG). The modulating effects of CRH (30 ng) on CeA activity were studied by infusion of CRH into the CeA in freely moving male RHA/Verh and RLA/Verh rats under stress-free conditions. Heart-rate and behavioural activities were repeatedly measured before, during and after local administration of CRH or vehicle, after which early gene product FOS immunocytochemistry and CRH-mRNA in situ hybridisation were carried out in selected brain areas. CRH infusion into the CeA caused a long lasting increase in heart-rate and behavioural activation in the RHA/Verh rats, leaving the RLA/Verh rats unaffected. As a result of CRH infusion, the number of FOS positive cells in the CeA and lPB of RLA/Verh rats was increased whereas an opposite response was found in the RHA/Verh rats. However, CRH into the CeA of the Roman rat lines induced no pronounced effects on FOS staining in the vlPAG and CRH mRNA levels in the CeA. These results indicate that the CRH system of the CeA, connected with the output brainstem areas, is differentially involved in cardiovascular and behavioural responses.

Keywords—RHA/RLA rats; Central amygdaloid nucleus; Heart-rate; Behaviour; Corticotropin-releasing hormone; FOS; CRH mRNA.

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INTRODUCTION

Corticotropin-releasing hormone (CRH) is a neuropeptide that has been widely associated with several behavioural and physiological aspects of stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991). In addition to its activating effects on the hypothalamus–pituitary–adrenal axis, intracerebroventricular (icv) injection of CRH produces elevations of heart-rate and blood pressure, as well as rises in plasma catecholamine and glucose levels (Bakke et al., 1990; Brown et al., 1982; Brown and Fisher, 1985; Fisher et al., 1983; Korte et al., 1993; Kurosawa et al., 1986). Furthermore, behavioural changes resembling effects of stress have been reported (Berridge and Dunn, 1987; Britton et al., 1982; Diamant and De Wied, 1991; Korte et al., 1993; Sherman and Kalin, 1988; Spadaro et al., 1990; Sutton et al., 1982; Veldhuis and De Wied, 1984). Among the brain sites responsible for the behavioural and cardiovascular effects of icv CRH, the central nucleus of the amygdala (CeA) has been implicated as an important locus. Icv CRH was found to induce FOS labelling in the CeA (Andreae and Herbert, 1993). Microinfusion of a low dose CRH (30 ng) into the CeA of male Wistar rats under stress-free conditions produces behavioural activation and a long lasting tachycardia without any rise in plasma catecholamines (Wiersma et al., 1993). This tachycardia has been suggested to be due to an inhibited cardiac parasympathetic output (Fisher, 1988; Fisher and Brown, 1991), exerted through the CeA (Wiersma et al., 1993, 1998). CRH induced effects after infusion into the CeA appear to be solely a CeA effect, as local application of CRH into the basolateral nucleus of the amygdala did not result in any activation at behavioural cardiovascular and neuroendocrine levels (Wiersma et al., 1998).

Infusion of a low dose of CRH (30 ng) into the CeA of male Wistar rats increased active behaviour in the conditioned defensive burying paradigm (Wiersma et al., 1996), whereas central blockade of its receptors by a CRH receptor antagonist (α-hCRH_{9-41}) inhibited this response (Korte et al., 1994). Immobility and burying behaviour in the defensive burying test has been suggested to represent a passive and active coping strategy, respectively, to that fearful situation (Korte et al., 1993). Several studies have shown that the Roman High-Avoidance line (RHA/Verh) displays an active coping response, characterised by high behavioural activity such as exploring and rearing, accompanied by a slight tachycardia (Bohus et al., 1987; Ferre et al., 1995; Koolhaas, 1994; Koolhaas and Bohus, 1991; Meerlo et al., 1997; Roozendaal et al., 1992). In contrast, RLA/Verh rats show a more passive coping response typically displaying behavioural immobility and grooming (Castanon and Mormede, 1994; Castanon et al., 1994; Driscoll et al., 1990; Driscoll and Battig, 1982; Willig et al., 1991) accompanied by either predominantly bradycardia (Bohus et al., 1987; Koolhaas, 1994; Roozendaal et al., 1993) or a tachycardia (D'Angio et al., 1988), depending on whether the stress is conditioned or not. As CRH connections from the CeA to the brainstem seem to be involved in active coping responses in Wistar rats (Wiersma et al., 1996), one may predict a differential modulation of the CeA output by CRH in rats which display different coping styles.

This hypothesis was tested in three ways. First, by investigating the behavioural and cardiovascular output of the CeA during and after local infusion of CRH in the genetically selected rat lines. Second, and in the same animals, CeA output was also studied by FOS immunocytochemistry on sections of the parabrachial nuclei (PB), one of the major projection areas of the CeA (Gray, 1993; Moga and Gray, 1985) and the periaqueductal gray (PAG). Since FOS is considered to be a marker for synaptically and/or transcriptionally active cells (Morgan and Curran, 1990), and icv CRH results in FOS labelling in the

PB as well as in the CeA (Andreae and Herbert, 1993), FOS immunocytochemistry was selected as a tool to reveal activated cells in the lateral PB, PAG and CeA. Finally, the possible differential CRH modulation of the CeA was studied by measuring CRH mRNA in the CeA by in situ hybridisation histochemistry both with and without intra-CeA CRH infusion. The in situ hybridisation technique was used as a marker of the number of CRH producing cells.

Based on behavioural, cardiovascular and histochemical observations, the present report reveals a differential effect of CRH infusion into the CeA between the different strains of Roman rats.

MATERIALS AND METHODS

Animals

Twenty-three RHA/Verh and 23 RLA/Verh (male, Wistar-derived) rats, weighing 280–380 g at the beginning of the experiment, were used. Animals were obtained from the breeding colony at the Animal Science Institute (Zurich, Switzerland) at the age of 5–8 weeks. The rats were housed in groups of five animals per cage and left undisturbed until the age of 14 weeks in a temperature controlled room (20 ± 1°C) with a 12-h light–dark cycle (lights on from 0800 to 2000h). Three days before surgery the animals were housed individually in perspex cages ($25 \times 25 \times 30$ cm) where food and water were available ad libitum. The experiments were carried out during the light period of the cycle (between 0900 and 1300h).

An additional number of three RHA/Verh and three RLA/Verh male rats, were used for basal levels of CRH mRNA in the CeA using in situ hybridisation. These rats were kept under complete stress-free conditions before perfusion.

Surgery

Operated animals were provided with bilateral permanent stainless-steel brain cannulae (outer diameter 0.3 mm, inner diameter 0.15 mm) for drug infusion, aimed just above the central nucleus of the amygdala (co-ordinates: 6.6 mm rostral to interaural, lateral 4.0 mm to the mid-line and ventral 6.3 mm below the dura) according to Paxinos and Watson (1982). The brain cannulae were permanently fixed to the skull by means of stainless-steel screws and dental cement.

In order to record the electrocardiogram (ECG) steel electrodes made of standard paper-clips were implanted transcutaneously, one between the scapulae and the other in the middle of the back (Bohus, 1974). The animals were kept under halothane anaesthesia in combination with N_2O and O_2 (ratio 2:1) during the entire surgical procedure.

Drug treatment

Synthetic rat/human CRH (CRF; Sigma, St. Louis, MO) was dissolved in artificial cerebrospinal fluid (aCSF) containing ascorbic acid (100 μg/ml). CRH was administered in a dose of 30 ng/rat per cannula, based upon the results of previous experiments (Wiersma et al., 1993, 1995). The vehicle was sterile artificial cerebrospinal fluid (aCSF) containing NaCl 127.64 mM, KCl 2.55 mM, $CaCl_2 \cdot 2H_2O$ 1.26 mM, and $MgCl_2 \cdot 6H_2O \cdot 0.93$ mM. All compounds were infused in a total volume of 1 μl in each brain cannula during a 7-min period.

ECG recording and analysis

The ECG of freely moving rats was monitored telemetrically by means of a miniature FM transmitter (EDB-ROY, HAREN, The Netherlands) as described earlier (Wiersma et al., 1993). The transmitter was connected to the transcutaneous electrodes and secured around the chest of the rat by means of a strap. The transmitted signals were received on a commercial FM receiver and stored on tape. For off-line analysis the recorded ECG samples were processed through a cardiotachometer pulse generator which generated square wave pulse at each R wave. The time between the onset of two consecutive pulses, the interbeat interval (IBI), was measured within the range of 100–220 ms by a personal computer (Olivetti M24). IBI's were recalculated in beats/min.

Behavioural measurements

Behaviour was recorded on the basis of the following criteria: resting/sleeping: inactive with eyes open or closed; immobility: completely motionless, absence of skeletal and vibrissae movements except those associated with respiration; exploring: locomotor activity to investigate any part of the home cage; grooming: wiping the fur with forepaws and tongue (washing); burying: pushing the bedding material with rapid movements of the snout and/or forepaws; rearing: sniffing in the air with both paws off the floor; sniffing: sniffing in the air with paws on the floor; eating: chewing food pellets or faeces. All behavioural elements were recorded by means of a keyboard operated microprocessor (EDB, Haren, The Netherlands). The duration and the frequency of these elements were recorded and expressed as the percentage of the total observation period.

Experimental procedures

The experiments were carried out in the animals' home cages under stress-free conditions. The rats were habituated to the strap holding of the transmitter, and to the infusion procedure, for at least a few hours during 3 days before the start of the experiments. The animals were tested after at least 10 days of recovery from surgery. Rats of the same line were divided into two groups receiving either vehicle (aCSF, 1 μl) or CRH (30 ng) only once. On the experimental days rats were connected to the infusion tubes and provided with the ECG transmitter 50 min before the start of their respective infusion into the CeA, and then returned to their home-cage and left undisturbed. The infusion tubes were counterbalanced by means of a pulley allowing the animals to move freely during the experiment. Time samples (1 min) of the behaviour and ECG were recorded simultaneously before ($t = -10$ and -1 min), during ($t = 3$ and 6 min), and after ($t = 8$, 10, 12, 17, 27, 47, and 67 min) the infusion period.

Histology

At different time points, 1.5, 2.0, 2.5 and 5.0 h after the start of the CRH or vehicle infusion, rats were randomised in these different groups. The rats were deeply anaesthetised with sodium pentobarbital (90 mg/kg IP) and perfused intracardially with cold saline followed by 400 ml of 4% paraformaldehyde, 3.8% Borax solution (pH 9.5). Brains were then removed and post-fixed in the same fixative for at least 2 h. Subsequently, the brains were dehydrated in a 30% sucrose in 0.1 M phosphate buffer (PB) for at least 48 h. Frozen sections (20 μm) were cut and cannula placement was determined on unstained sections. Sections were stored in cryoprotectant solution at -20°C until processing forFOS immunocytochemistry and CRH mRNA in situ hybridisation histochemistry. The

cryoprotectant solution consisted of 0.5 l of polyethylene glycol and 300 g sucrose dissolved in the same volume of 0.2 M PB.

Immunohistochemistry

Every third section was stained for FOS-like immunoreactivity by the avidin–biotin–peroxidase technique. Prior to the first antibody incubation, sections were immersed for 10 min in 0.3%·H_2O_2 in 0.01 M phosphate-buffered saline (PBS) to exhaust endogenous peroxidase activity. The sections were then incubated for 1 h at room temperature (RT) in 5% normal rabbit serum (NRS) to suppress non-specific antibody binding. The sections were washed 2 × for 10 min in PBS, then incubated for 48 h with the primary antibody of a sheep-anti-FOS antibody solution (1:2000; Cambridge Research Biochemicals, UK) in PBS and 0.3% Triton-X at 4°C. This antibody has been raised against the first 16 amino-acids derived from a conserved region of both the mouse and human FOS-protein. After first antibody incubation, the sections were rinsed 2 × in PBS and incubated for 1 h in rabbit anti-sheep antibody (Pearce rabbit anti-sheep IgG), biotin conjugated, 1:800 in PBS and 0.3% Triton-X, washed again and followed by 1 h incubation in avidin–biotin complex (Vectastain ABC kit 0.4% in PBS). This procedure was repeated once more before the sections were stained by the reaction product of diaminobenzidine tetrahydrachloride (DAB) and hydrogen peroxide H_2O_2 (respectively 1 mg/ml and 0.0075%) in a nickel ammoniumsulfate solution (50 g/l 0.2 M sodium acetate buffer, pH 6.0). Stained sections were mounted, dried overnight, dehydrated, cleared in xylene and coverslipped with DPX mountant (BDH-Pool, UK).

In situ hybridisation histochemistry

The CRH peptide coding region containing template was linearised with Hind III. SP polymerase was used to synthesise roboprobe complementary to rat CRH mRNA. Sections were washed 2 × in PBS and mounted on pre-coated poly L lysine slides. After they had been vacuum dried overnight, the sections were post-fixed by incubation in a 4% paraformaldehyde, 3.8% Borax solution (pH 9.5) for 30 min. Pre-hybridisation steps included 0.01 mg/ml proteinkinase K digestion for 30 min at 37°C, acetylation for 10 min at room temperature, and dehydration in a graded series of alcohol solutions. Additional sections were vacuum dried for at least 2 h before the hybridisation solution was applied. The hybridisation solution contained 50% formamide, 10% dextran sulfate, 0.3 M NaCL, 1 × Denhardt's solution, 10 mM Tris (pH 8.0), 1 mM EDTA, 500 μg/ml tRNA, 10 mM dithiothreitol (DTT) and the probe at the concentration of 10^7 cpm/ml. To the sections on each slide, about 80 μl of hybridisation solution was applied, a coverslip then being placed over the slide and sealed around the edges with liquid DePeX mountant. The slides were incubated on a covered slide-warming tray at 55°C for 16 h. After the hybridisation, the cover-slips were removed, and the sections were rinsed in NaCl/Na citrate (SSC) and digested with ribonuclease A (0.02 mg/ml) for 30 min at 37°C. After this, the slides were rinsed in descending concentrations of SSC at room temperature, incubated in 0.1 × SSC at 60°C for 30 min, dehydrated in series ethanol and dried under vacuum at room temperature for at least 2 h. All SSC rinse solutions following RNAase treatment contained 1 mM DTT. Sections were delipilised in 95% ethanol (5 min), followed by absolute ethanol (3 × 5 min), xylene (10 min), fresh xylene (30 min). They were vacuum dried before being dipped in Kodak autoradiography emulsion (NTB-2). After an exposure time of 1 month the slides were developed, fixed and counter stained with 0.5% Cresyl Violet.

Quantification

The numbers of FOS-positive cells in the CeA, lPB and PAG were counted using a light microscope on alternating sections from each rostrocaudal level. Cells were selected to be FOS-positive on basis of their grain densities. The numbers of FOS-positive cells in the CeA were counted on alternating sections at the levels 6.2, 6.44, 6.7, 6.88 and 7.2 mm from intraaural according to Paxinos and Watson (1982). A minimum of eight sections were counted for each animal. The numbers of FOS positive cells in the lPB were counted on alternating sections at the levels -0.8, -0.68, -0.3, -0.16 and $+0.2$ mm from intraaural. A minimum of six sections were counted for each animal. The numbers of FOS positive cells in the PAG were counted on alternating sections at the levels 0.2, 0.7, 1.2 and 1.7 from intraaural according to Paxinos and Watson (1982). A minimum of six sections were counted for each animal.

The numbers of CRH mRNA positive cells in the CeA were counted using a low power dark-field microscope on alternating sections from each rostrocaudal level. Cells were selected to be CRH-positive on basis of their grain densities. The numbers of CRH mRNA positive cells in the CeA were counted on alternating sections at the levels 6.2, 6.7, 6.88 and 7.2 mm from intraaural according to Paxinos and Watson (1982). Due to difficulties in the staining procedure and to problems with the tissue, which, as a result of the cannula tracts, resulted in tearing in the CeA areas of the slices of some animals, the CRH mRNA counting of the groups of 1.5, 2.0 and 2.5 h were taken together. A minimum of seven sections were counted for each animal.

Basal CRH in situ hybridisation in the PVN and CeA

In non-treated animals, the numbers of CRH parvocellular cells of the paraventricular nucleus were counted using a low power dark-field microscope on alternating sections from each rostrocaudal level. A minimum of four sections of the PVN were counted for each animal. Cells were selected to be CRH positive on basis of their grain densities. The numbers of CRH mRNA positive cells in the CeA were counted on alternating sections at the levels 6.2, 6.7, 6.88 and 7.2 mm from intraaural, also according to Paxinos and Watson (1982). A minimum of seven sections were counted for each animal.

Statistics

The results are presented as mean $\pm$ SEM. Cardiac and behavioural data were evaluated using an analysis of variance with repeated measures (MANOVA) and followed by the Mann-Whitney U-test. The immunocytochemistry data were evaluated using a multiple analysis of variance followed by post-hoc Student t-test. A probability level of $p < 0.05$ was taken as significant for all tests.

RESULTS

Histology

Following histological examination, five out of the 46 animals used had to be excluded from further analysis because of improper bilateral cannula placement. The cannula tips had to be localised just above or entering the dorsal edge of the CeA.

Cardiac responses

Fig. 1 shows the change in heart-rate before, during and after infusion of 30 ng of CRH or aCSF into the CeA, compared with basal values of heart-rate ($t = -10$ and -1 min). No significant differences were found in basal heart-rate values of all the groups (RLA-CSF, 386.3 ± 7.8; RLA-CRH, 383.5 ± 12.8; RHA-CSF, 385.8 ± 16.7; RHA-CRH, 362.5 ± 18.7). Local infusion of 30 ng of CRH into the CeA produced an increase in heart-rate, only in the RHA/Verh rats. The tachycardia started during the infusion and lasted for at least 30 min after the start of the infusion. Locally applied CRH (30 ng) into the CeA of RLA/Verh rats, as well as local infusion of vehicle (CSF) into the CeA of both RHA/Verh and RLA/Verh animals did not induce any change in heart-rate. The ANOVA showed a significant main effect of treatment in RHA rats ($F_{1,7} = 5.2$, $p < 0.05$). Analysis of the separate time samples revealed significant differences in heart-rate response between the RHA-CRH treated animals and the RHA-CSF treated group at $t = 6$ min ($p < 0.05$), $t = 8$ min ($p < 0.05$), $t = 10$ min ($p < 0.05$) and at $t = 12$ min ($p < 0.05$).

Behavioural responses

Resting/sleeping were the major behavioural elements displayed by the animal during the experiments (Fig. 2). Resting in both the RHA/Verh and RLA/Verh rats treated with vehicle remained at a constant level throughout the experiment. Infusion of CRH into both RHA/Verh and RLA/Verh treated rats decreased resting. This decrease was more-pronounced in the RHA-CRH treated animals than in the RLA-CRH treated animals, the slight CRH induced reduction of resting in the latter never reaching significance. The reduction in resting in the RHA-CRH rats lasted for 17 min. Analysis of variance revealed significant treatment effect ($F_{3,25} = 5.104$, $p < 0.01$) and treatment × time interaction

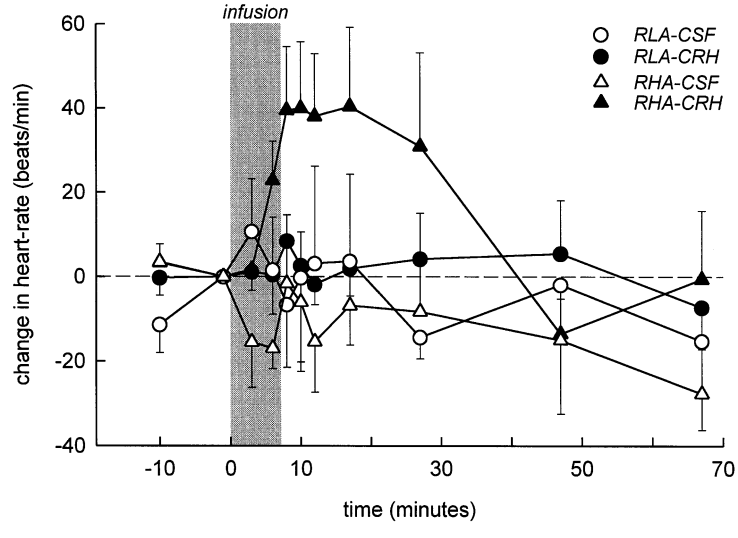

Fig. 1. Changes in heart-rate ($\pm$ SEM) as a consequence of infusion of CRH (30 ng in 1 μl) or artificial-CSF (1 μl) for 7 min at $t = 0$ min into the CeA of RHA/Verh (RHA) and RLA/Verh (RLA) rats under stress-free conditions. The infusion period is indicated by the grey bar. Vehicle-group: RLA-CSF, $n = 5$; RHA-CSF $n = 5$; RLA-CRH $n = 9$; RHA-CRH $n = 6$.

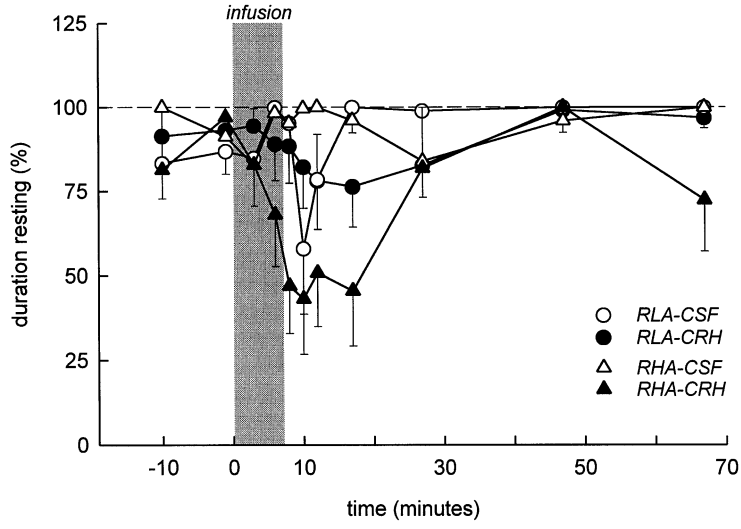

Fig. 2. Relative duration of resting/sleeping behaviour before, during and after microinfusion of CRH (30 ng in 1 μl) or aCSF (1 μl) at $t = 0$ min into the CeA of RHA/Verh (RHA) and RLA/Verh (RLA) under stress-free conditions. The infusion period is indicated by the grey bar. Data are expressed as averages $\pm$ SEM. Vehicle-groups (CSF): RLA-CSF $n = 6$; RHA-CSF $n = 6$. CRH-groups: RLA-CRH $n = 9$; RHA-CRH $n = 9$.

($F_{4,100} = 2.265$, $p < 0.05$) between all the groups. The treatment effect was significant for the RHA/Verh groups ($F_{1,13} = 11.137$, $p < 0.01$) and a tendency was found between the RLA-CRH and RHA-CRH treatment groups ($F_{1,16} = 4.053$, $p < 0.058$). Significant treatment $\times$ time interactions were only found between RHA-CSF and RHA-CRH ($F_{4,52} = 3.732$, $p < 0.01$). Comparison of the time samples showed significant differences in the RHA rats at $t = 10$ min ($p < 0.01$), $t = 12$ min ($p < 0.01$) and $t = 17$ min ($p < 0.05$).

The behavioural elements most frequently displayed by animals when not resting, were rearing and exploring. The occurrence of these and other behaviours, i.e. immobility, grooming, burying, sniffing and eating, did not differ significantly between the treatments and lines of rats (data not shown).

FOS immunocytochemistry

The results of FOS immunocytochemistry in the brain structures of animal of the different treatment groups are shown in Fig. 3. There was no difference between the number of FOS-positive cells measured at 1.5 and 2 h, which were therefore combined. Comparison of the number of FOS-positive cells in aCSF and CRH-treated animals in both the CeA and the lPB did not reveal any significant within-line differences (both CeA and lPB, $F_{3,17} = 2.01$, $p = 0.14$). There was, however, a tendency for RLA-CSF treated animals to display less FOS-positive cells in the CeA ($p = 0.06$) and the lPB ($p = 0.15$) than RHA-CSF treated rats. CRH infusion decreased the number of FOS positive cells in the CeA ($p = 0.012$) and lPB ($p = 0.09$) of RHA/Verh rats. In contrast, CRH infusionincreased the number of FOS positive cells in the CeA ($p < 0.05$, Student's t-test) but not in the lPB of RLA rats.

FOS counting in the PAG revealed some differences only in the ventrolateral column of the PAG (vlPAG). CRH microinfusion into the CeA increased the number of FOS-positive cells in the vlPAG of both rat lines, with a tendency toward significance being found between the RHA-CSF and the RHA-CRH groups ($p = 0.07$). No differences were found in the dorsolateral column of the PAG.

The rats perfused at 2.5 and 5 h showed a marked reduction in the total amount of FOS positive cells both in the CeA and the lPB compared with the rats perfused at 1.5 and 2 h. However, no differences between the lines and treatments were found.

CRH mRNA in situ hybridisation histochemistry

A significant difference in CRH mRNA expression was found between the rat lines in the hypothalamic PVN under basal conditions (mean number of silver grains: 72.1 ± 6.3 in RHA/Verh rats vs 50.5 ± 4.5 in RLA/Verh rats: $F_{1,4} = 7.65$, $p < 0.05$). This difference did not reach significance in the CeA (30.4 ± 2.6 vs 24.0 ± 2.6: $F_{1,4} = 3.06$, $p > 0.10$).

The number of CRH mRNA positive cells in the CeA of the Roman rat lines with CRH or aCSF locally into the CeA is shown in Fig. 4. There was no significant line and treatment effect.

DISCUSSION

The major finding of the present study is that local CRH microinfusion into the CeA of RHA/Verh and RLA/Verh rats resulted in distinctly different behavioural, physiological and neurobiological responses under stress-free conditions.

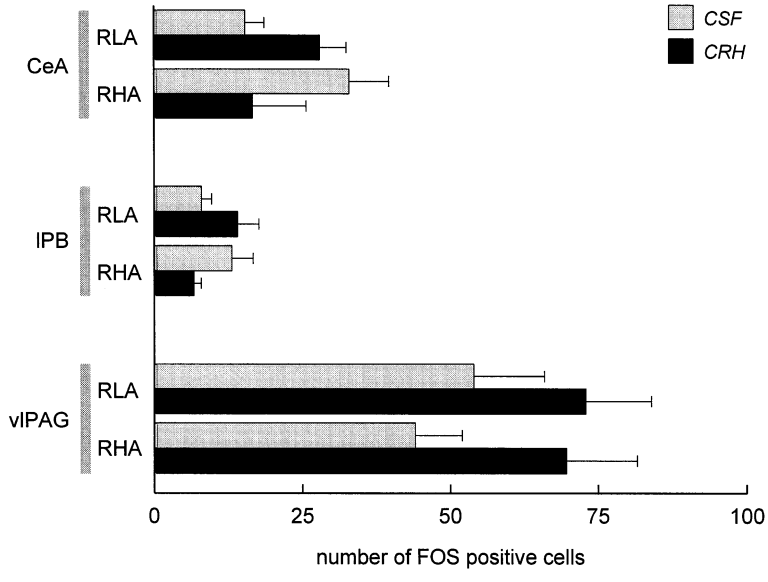

Fig. 3. Amount of FOS positive cells in the CeA (upper panel), lPB (middle panel) and vlPAG (lower panel), 1.5 and 2.0 h after the infusion of aCSF (1 μl) or CRH (30 ng in 1 μl) into the CeA of RLA/Verh (RLA) and RHA/Verh (RHA) rats under stress-free conditions. Data are expressed as averages $\pm$ SEM. Vehicle-groups (CSF): RLA-CSF $n = 5$; RHA-CSF $n = 7$. CRH-groups: RLA-CRH $n = 5$; RHA-CRH $n = 4$.

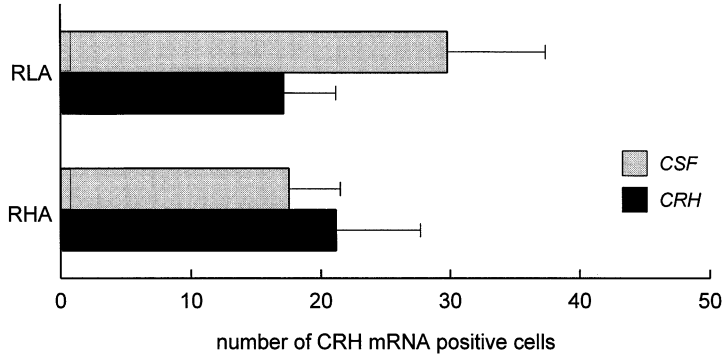

Fig. 4. Amount of CRH mRNA positive cells in the CeA 2 h after the infusion of aCSF (1 μl) or CRH (30 ng in 1 μl) into the CeA of RLA/Verh (RLA) and RHA/Verh (RHA) rats under stress-free conditions. Data are expressed as averages $\pm$ SEM. Vehicle-groups (CSF): RLA-CSF $n = 7$; RHA-CSF $n = 7$. CRH-groups: RLA-CRH $n = 7$; RHA-CRH $n = 6$.

RHA/Verh rats responded to this treatment with an increase in heart-rate which lasted for at least 17 min. This was accompanied by decreased resting/sleeping indicating an increase in overall behavioral activation. The same treatment induced only a slight behavioral activation and had no effect on heart-rate in RLA/Verh rats. Fos immunocytochemistry also revealed a differential response between the two rat lines. The number of FOS positive cells was smaller in the CeA and the lPB of RLA/Verh rats in comparison to RHA/Verh animals. CRH infusion resulted in contrasting consequences, inducing an increase in FOS positive cells in the RLA/Verh animals, and a decrease in RHA/Verh rats. In situ hybridisation of CRH mRNA expression under basal conditions revealed between the rat lines a difference in the PVN, but not in the CeA. However, vehicle treated RLA/Verh rats had slightly more CRH mRNA positive cells in the CeA compared to the vehicle treated RHA/Verh rats. CRH mRNA expression in the CeA was not affected by CRH infusion in the RHA/Verh animals, while in the RLA/Verh rats a small decline was observed. These results all together support the hypothesis that the CRH system in the CeA is differentially involved in cardiovascular and behavioural control under stress-free conditions in lines of rats having different coping styles.

The cardiovascular and behavioural results of the present study are in agreement with earlier findings demonstrating that CRH administration into the CeA induces a tachycardia together with behavioural activation in Wistar rats (Wiersma et al., 1993). The behavioural activation and tachycardia seen after infusion of 30 ng CRH into the CeA of Wistar rats used in the previous study was not accompanied by a rise in plasma catecholamine, suggesting a diminished parasympathetic cardiovascular output (Wiersma et al., 1993). Similar cardiovascular and behavioural effects were observed only in RHA/Verh rats but not in RLA/Verh rats which only displayed a slight behavioural activation in response to CRH under stress-free conditions.

Although the CRH induced tachycardia and behavioural activation in the Wistar-derived RHA's showed the same temporal pattern previously observed in Wistars rats (Wiersma et al., 1993), the amplitude of these effects was much larger in the RHA/Verh animals. This suggests that the CRH-responsive system in the CeA is more sensitive in

RHA/Verh rats than in the Wistar rats used, and that the RLA/Verh animals are even less sensitive to infusion of the same dose of CRH.

Differences in sensitivity to various stressors between the RHA/Verh and RLA/Verh rats have been observed in a number of studies (Driscoll and Battig, 1982; Driscoll et al., 1990; Escorihuela et al., 1995). For example, RHA/Verh rats are generally more active in various novel environments and show a smaller stressor-induced increase in ACTH as well as corticosterone, prolactin, and other hormones than do RLA/Verh rats (Aubry et al., 1995; Castanon and Mormede, 1994; Castanon et al., 1995; Gentsch et al., 1981, 1982; Walker et al., 1989). The two lines display differences in pituitary–adrenal responses to exogenous CRH (Walker et al., 1989, 1992) and vasopressin (Roozendaal et al., 1992). Earlier studies on the effects of infusion of various neuropeptides into the CeA of RHA/Verh and RLA/Verh rats also revealed distinct different behavioral and cardiovascular responses between the two lines (Roozendaal et al., 1993). Infusion of vasopressin into the CeA resulted in behavioral and cardiovascular responses in RLA/Verh but not in RHA/Verh rats (Roozendaal et al., 1992).

The differential sensitivity of Roman lines to CRH infusion into the CeA can be explained by differences at the level of receptor or post-receptor mechanisms. The mechanisms for these differences remain to be elucidated.

Quantification of FOS positive cells in the CeA and lPB revealed that CRH infusion into the CeA induced differences in the amount of activated cells in the CeA and lPB between the two lines. Remarkably, the changes in the number of FOS positive cells correlated negatively with the behavioural and cardiovascular effects of CRH. This can be interpreted to suggest that exogenous CRH in the CeA inhibits some of the autonomic regulating brainstem areas involved especially in the parasympathetic regulation.

In basal situations RHA/Verh and RLA/Verh rats appeared to have the same amount of CRH mRNA positive cells in the CeA, while in the PVN the RHA/Verh rats showed a greater amount of positive cells than do their RLA/Verh counterparts. This is in contrast with data from Aubry and colleagues, who found no significant difference in CRH mRNA level of the PVN (Aubry et al., 1995). Our results show that these lines genetically selected on the basis of different behavioural responses, differ in basal gene expression levels for hypothalamic CRH but not for amygdalar CRH. CRH mRNA positive cells in the CeA was not affected by CRH infusion in the RHA/Verh animals and tended to decline in RLA/Verh rats.

The results of FOS immunocytochemistry and CRH mRNA reveal that CRH infusion into the CeA modulated the neuroanatomical level, the CRH CeA output to the lPB and the vlPAG, in a differential way. This difference between the lines may play an important role in the underlying mechanisms that are responsible for their differential behavioural responses in stress situations.

In conclusion, the present results reveal that exogenous CRH applied to the CeA under stress-free conditions, results in distinct different behavioural, cardiovascular and neurobiological responses in two rat lines genetically selected on the basis of divergent behavioural patterns.

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