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Endogenous corticotropin-releasing hormone inhibits conditioned-fear-induced vagal activation in the rat

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Abstract

The role of the endogenous corticotropin-releasing hormone (CRH) system in the regulation of heart rate, PQ interval (a measure of vagal activity), gross activity and release of adrenocorticotropic hormone (ACTH), noradrenaline and adrenaline into the blood during conditioned fear was studied in freely moving rats. Intracerebroventricular (i.c.v.) infusion of α-helical CRH-(9-41) (10 μg/3 μl), a non-selective CRH receptor antagonist, under resting conditions had no significant effect on gross activity, heart rate and PQ interval, indicating that α-helical CRH at this dose was devoid of agonist effects. Conditioned fear was induced by 10 min forced exposure to a cage in which the rat had experienced footshocks (5 × 0.5 mA × 3 s) 1 day before. Conditioned-fear rats showed freezing behaviour, associated with an increase in heart rate, PQ interval, noradrenaline and adrenaline, indicating that the conditioned-fear-induced cardiac effects were the result of coactivation of the sympathetic and parasympathetic nervous system. The i.c.v. pre-treatment of rats with α-helical CRH significantly reduced the conditioned-fear-induced tachycardiac and ACTH response, and enhanced the increase in PQ interval, without affecting the noradrenaline and adrenaline response. These results suggest that endogenous CRH reduces the vagal response to conditioned-fear stress in rats. To test this, rats were pre-treated with atropine methyl nitrate (0.3 mg/kg, subcutaneously; s.c.), a peripherally acting cholinergic receptor antagonist. This resulted in a complete blockade of the α-helical CRH-induced decrease in heart rate response and increase in PQ interval. From these findings, it is concluded that endogenous CRH in the brain inhibits vagal outflow induced by emotional stress.

Keywords: Stress; Behaviour; Autonomic nervous system

1. Introduction

Endogenous corticotropin-releasing hormone (CRH) acts within the central nervous system to mediate behavioural, autonomic and hormonal responses to various stressors (Brown et al., 1985; Kalin and Takahashi, 1990; Cole and Koob, 1991; Morimoto et al., 1993; Menzaghi et al., 1994). However, the exact role of the sympathetic and parasympathetic branches of the autonomic nervous system in CRH-induced cardiac responses to stress has so far not been established. Brown et al. (1985) reported that intracerebroventricular (i.c.v.) α-helical CRH-(9-41) blunted the stress-induced increase in plasma adrenaline in rats, suggesting that endogenous CRH is involved in the stress-induced adrenomedullary response. The stress-induced increase in plasma noradrenaline, which represents an index of sympathetic nervous system activity (Lake et al., 1976), was, however, not affected by blockade of the endogenous CRH system. Others have demonstrated that α-helical CRH blocked the CRH-induced plasma noradrenaline and adrenaline response (Brown et al., 1985). So far, however, no direct evidence has been provided that the endogenous CRH system is involved in stress-induced elevations in sympathetic nervous system activity.

The involvement of the parasympathetic nervous system in the stress response is even less clearer, as this division of the autonomic nervous system has received less attention in stress research (Porges, 1992; Wiersma et al., 1993; Bohus et al., 1996). An important reason for this is the difficulty in obtaining direct information on vagal activity in conscious, freely moving animals. Wiersma et al. (1993)

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reported that central CRH might play a role in the vagal response to stress as they showed that CRH infusion in the central nucleus of the amygdala in rats under resting conditions increased HR without affecting plasma catecholamine levels, suggesting inhibition of cardiac vagal outflow. Fisher (1989) and Fisher and Brown (1991) suggested that exogenous CRH can inhibit the baroreceptor reflex by a suppression of vagal cardioinhibitory neurons, resulting in an increase of both blood pressure and heart rate. Wang et al. (1996) reported that i.c.v. CRH inhibited restraint-induced expression of Fos in the dorsal motor nucleus of the vagus in rats. Stress-induced activation of this brainstem nucleus, from which vagal efferent fibres originate, suggests that the parasympathetic nervous system is activated by stress. The authors concluded that parasympathetic nervous system outflow is reduced by exogenous CRH during stress.

In the present study, the involvement of the parasympathetic nervous system in endogenous CRH-mediated responses during stress was investigated by using the PQ interval of the electrocardiogram as a measure of vagal activity (Croiset et al., 1994; Nijsen et al., 1998a,b). The PQ interval is defined as the interval between the beginning of the P wave and the beginning of the QRS complex in an electrocardiogram, and mainly represents the so-called atrioventricular conduction time. The lengthening of the PQ interval is influenced by both the sympathetic and parasympathetic nervous systems. Elongation of the PQ interval can be caused by an increase in vagal and/or a decrease in sympathetic outflow (Levy and Zieske, 1969; Suzuki et al., 1991; Penz et al., 1992; Croiset et al., 1994). In general, stress induces sympathetic activation (De Boer et al., 1990; Korte et al., 1992). Thus, a stress-induced increase in PQ interval can be attributed to vagal activation. When sympathetic nervous system activity is also monitored, for instance by determination of plasma noradrenaline, sympathetic and vagal contribution can be further distinguished (Nijsen et al., 1998b).

The aim of this study was to examine the role of the endogenous CRH system in the autonomic response during emotional stress. To that end, rats were pre-treated i.c.v. with α-helical CRH and exposed to conditioned fear. In order to differentiate the contribution of the sympathetic and parasympathetic nervous systems to the stress-induced cardiac response, heart rate and PQ interval, as well as plasma levels of noradrenaline and adrenaline, were determined.

2. Materials and methods

2.1. Animals and housing

Naive male albino rats of an outbred Wistar strain (U:WU), weighing 250–300 g at the beginning of the experiments, were used. Rats were housed individually in Macrolon cages ($23 \times 17 \times 14$ cm^3) containing a layer of woodshavings, under conditions of constant ambient temperature (21 ± 1°C), constant humidity and normal light/dark rhythm (with lights on from 0700 to 1900 h). After surgical implantation of a telemetric transmitter or cannulation of the jugular vein, the animals were housed individually in Plexiglas cages ($25 \times 25 \times 40$ cm^3) under pre-surgical conditions. For the conditioned fear model, a $30 \times 32.5 \times 38.5$ cm^3 shock box was used, with stainless steel walls, a Plexiglas door and a grid floor made of 2.5-mm brass rods spaced 1.0 cm apart. Food (complete laboratory chow: Hope Farms, Woerden, the Netherlands) and water were accessible ad libitum throughout the experiment.

2.2. Surgery

One group of rats was equipped with telemetric devices to study gross activity, heart rate and PQ interval. Another group of rats was provided with a cannula in the jugular vein to determine plasma noradrenaline and adrenaline concentrations. All rats received a guide cannula implanted into the lateral brain ventricle for i.c.v. infusion. The guide cannula consisted of a titanium needle (outer diameter (o.d.), 0.4 mm; inner diameter (i.d.), 0.2 mm) closed by a tungsten stylet. Operations were performed under fentanyl/fluanisone anaesthesia (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium; 0.1 ml/100 g body weight (BW), i.m.) and Midazolam hydrochloride (Dormicum®, Hoffman-LaRoche, Mijdrecht, The Netherlands; 0.1 ml, i.p.) as a muscle relaxant. Before the muscle relaxant was injected, the analgesic effect of fentanyl anaesthesia was tested in the rat by checking for the absence of pedal and corneal reflexes. Total absence of the pain response normally appeared after 10 min, and then the muscle relaxant was injected.

Surgery was performed in a sterile laminar flow cabinet to minimalise risk of infection. Telemetric transmitters were implanted in the abdominal cavity, according to the procedure described by Wan et al. (1990). A small longitudinal incision was made on the linea alba at the anterior of the abdomen. Two electrodes, originating from the top of the transmitter, were guided subcutaneously (s.c.) on either side of the thorax; the electrode tips were sutured into position to obtain a bipolar electrocardiogram signal. Changes in position of this transmitter relative to the receiver reflect the amount of gross activity.

For blood sampling procedures, the animals were cannulated according to the method of Steffens (1969) with some modifications. A sterile silicon cannula (Silastic®, Dow Corning, Midland, MI, USA), filled with a 50 IU/ml heparin solution (0.9% NaCl, containing 50 IU/ml heparin), was inserted into the external jugular vein and passed down near the entrance of the atrium. The other end of the cannula was guided underneath the skin towards the skull, connected to a stainless steel tube and attached to the

skull with dental cement. The end of the cannula was filled with 0.05 ml PVP (50% PolyVinylPyrrolidonum in Millipore water, containing 50 IU/ml heparin), a viscous solution, to keep the cannula patent. Post-operatively, the animals received 0.1 mg/kg of the long-acting opiate analgesic Buprenorfine hydrochloride (Temgesic®, Reckitt & Colman, Kingston-upon-Hull, UK; 0.1 ml, s.c.).

Animals were allowed to recover from surgery for 10 days in the experimental room. During the recovery period, the animals were handled every day for weighing and habituation purposes. Rats were accustomed to blood sampling procedures (twice before the experiment). After the experiment, all rats were killed by an overdose (0.5 ml) of pentobarbital (160 mg/ml), dissected and macroscopically inspected for infections; signs of infection were not found in any animal.

2.3. Telemetry

The telemetry system consisted of small wireless transmitters, model TA11CTA-F40 (Data Sciences, St. Paul, MN, USA), and receivers, model RLA1020 (Data Sciences). Digital data, electrocardiograms and gross activity were transmitted from the receiver to a DataQuest IV data acquisition system (Data Sciences). Gross activity was monitored by digital pulses that indicate the occurrence of an event of movement activity. The rate at which the events were polled for changes was 64 Hz. These pulses were counted by the DataQuest IV system and totaled over the sampling period. Then, the system scales the raw values to correspond to counts per minute. Mean heart rate was automatically calculated from electrocardiograms. The PQ interval is defined as the interval between the beginning of the P wave and the beginning of the QRS complex. PQ intervals were automatically analysed from 10-s electrocardiogram recordings and were averaged by a special software program, PhysioStat PS1000 (Data Sciences).

In experiment 1, a 10-s sampling period with an interval of 5 min was used to record electrocardiograms and gross activity during a 60-min recording period in the home cage after drug infusion. In experiments 2 and 4, a 10-s sampling period with intervals of 3 min was used during a 30-min recording period in the home cage just before (baseline) and during a 60-min recording period in the home cage, just after (post-treatment) conditioned fear. A 10-s sampling period with intervals of 30 s was used to record electrocardiograms and gross activity during conditioned fear. To simplify Figs. 1 and 4, data points at 1-min intervals instead of 30 s are presented during 10-min conditioned fear. The data from two samples taken at 30-s intervals have been pooled to produce each data point. Data points at 15-min intervals, instead of 3 min, are presented after conditioned fear. The data from five samples, taken at 3-min intervals, have been pooled to produce each data point.

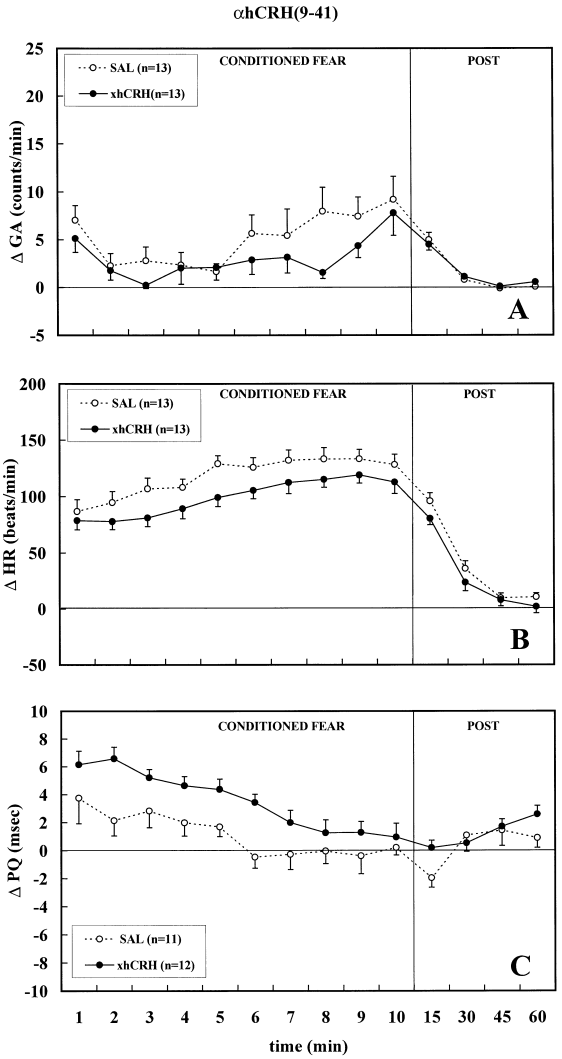

Fig. 1. Changes in gross activity (GA) (A), heart rate (HR) (B) and PQ interval (C) in α-helical CRH-treated rats (xhCRH) or saline-treated rats (SAL) during and after 10 min conditioned fear. α-Helical CRH (10 μg/3 μl) or saline was infused i.c.v. from $t = -15$ to -9 min. The vertical line marks the end of conditioned fear stress. Data are presented as mean ± SEM. Equal gross activity levels in both treatment groups are represented as one filled circle. Data that show no error bars represent average values with minimum SEM.

2.4. Adrenocorticotropic hormone (ACTH) determination

ACTH concentrations in plasma were determined by radioimmunoassay (RIA). Plasma ACTH was determined in duplicate according to the procedure described by Van Oers and Tilders (1991) using a specific rabbit antiserum directed to the mid-portion of ACTH (code Ft 8514), kindly donated by Dr. G.B. Makara (Budapest, Hungary). Synthetic human ACTH-(1-39) (Peninsula Laboratories, Belmont, CA, USA) was used as standard, and ^{125}I-labelled ACTH-(1-39) (Iodogen® method) as tracer. Sample dilution curves paralleled the standard curve. The sensitivity of the assay, calculated at $B/B_0 = 0.9$, was 10 pg/ml plasma (0.5 pg/tube). Intra- and interassay variations were 5% and 8%, respectively.

2.5. Catecholamine determination

Catecholamine levels were determined by high-performance liquid chromatography (HPLC) based on isocratic reversed-phase chromatographic separation in combination with amperometric detection. All determinations were performed on a Separations model GT-103 liquid chromatograph (Separations Analytical Instruments, H.I. Ambacht, the Netherlands) equipped with an automatic sampler and injector in conjunction with a peak column (Inertsil®, G.L. Sciences, Tokyo, Japan; packing: Inertsil ODS-2, particle size: 5 μm) and an amperometric detection system consisting of auxiliary electrodes and an Ag–AgCl reference electrode. The resulting signal was processed by an Axiom Chromatography model 727 chromatography data system. Both the column and the detector cell were placed in the column oven of the liquid chromatograph.

To the plasma samples (100 μl), 10 mg aluminum oxide (Recipe Chemicals and Instruments, München, Germany), 11 μl internal standard (3,4-dihydroxybenzylamine (Sigma-Aldrich, Zwijndrecht, the Netherlands); 5 μM dissolved in water) and 300 μl 2 M Tris buffer D (Recipe Chemicals and Instruments) were added. After mixing, the supernatant (110 μl) was removed and the aluminum oxide was washed three times with 400 μl Tris buffer (Recipe Chemicals and Instruments). Glacial acetic acid solution (Merck, Darmstadt, Germany; 110 μl) was used to elute the catecholamines from the aluminum oxide. From this eluate, 90 μl was injected on HPLC. In each run, standard samples (100 μl) were included containing noradrenaline and adrenaline (Sigma-Aldrich; 5 μM dissolved in water) and BA. Data of unknowns were calibrated to the standards, and expressed as picogram per milliliter plasma. The sensitivity of the assay was 1 pg/ml sample.

2.6. Drug treatment

α-Helical CRH-(9-41) (Bachem Feinchemikalien, Bubendorf, Switzerland), a non-selective CRH receptor antagonist, was dissolved in saline and was i.c.v. infused (10 μg/3 μl/6 min). For stress-free i.c.v. infusion, 1 h prior to testing, rats were connected to a long (1.5 m) fused-silica line (i.d. 75 μm, o.d. 150 μm; Composite Metal Services, Hallow, UK), guided by a polyethylene tube (i.d. 0.28 mm, o.d. 0.61 mm; Portex, Hythe, Kent, UK), which was attached to a syringe in a microinfusion pump (Harvard Apparatus, MA, USA). The end of the fused-silica line was filled with 5 μl saline or α-helical CRH solution, from which 3 μl was infused i.c.v. The rest of the line was filled with saline. To avoid dead space, the fused-silica line was inserted into the i.c.v. guide cannula until the tip of the fused-silica line reached the end of the guide cannula in the brain. One hour prior to blood sampling, rats were connected with a long (1 m) polyethylene tube (i.d. 0.76 mm, o.d. 1.22 mm), which was attached to a syringe for manual blood withdrawal. During i.c.v. infusion or basal blood withdrawal, all rats were asleep, indicating that our long-line technique is a stress-free method for infusion or blood sampling.

Atropine methyl nitrate (Sigma-Aldrich), a muscarinic cholinergic receptor antagonist that does not cross the blood–brain barrier, was dissolved in saline and was injected s.c. (0.3 mg/kg BW) in a constant volume of 0.25 ml. In a previous study (Nijsen et al., 1998b), we demonstrated that the cardiac effects (increase in heart rate and shortening of PQ interval) of atropine methyl nitrate administered s.c. to rats in their home cage occur almost immediately, reach their maximum well within 30 s, remain steady for more than 10 min, and last for at least 60 min after treatment. Similar dynamics of the response to atropine were observed when rats were exposed to conditioned-fear stress (Nijsen et al., 1998b).

2.7. Experimental design

2.7.1. Experiment 1: effect of i.c.v. α-helical CRH on gross activity, heart rate and PQ interval under resting conditions

Baseline heart rate, PQ interval and gross activity of individual, single-housed rats were telemetrically recorded for 30 min under resting conditions. Subsequently, rats were infused i.c.v. with saline ($n = 8$) or α-helical CRH ($n = 8$) and heart rate, PQ interval and gross activity were recorded for another 60-min period. Throughout the experiment, rats remained in their home cages.

2.7.2. Experiment 2: effect of i.c.v. α-helical CRH on gross activity, heart rate and PQ interval during conditioned fear

On day 1 of the experiment, individual, single-housed animals with implanted transmitters were transferred one by one to a novel shock box where they remained for 10 min. A group of 26 rats received 5×0.5 mA $\times$ 3 s footshocks given at random intervals throughout the 10-min period starting 30 s after entry. On day 2, baseline heart rate, PQ interval and gross activity of rats were recorded in the home cage for 30 min under resting conditions, and then rats were i.c.v. infused with saline ($n = 13$) or α-helical CRH ($n = 13$). Fifteen minutes after i.c.v. infusion, the procedure from day 1 was repeated, except that none of the animals received any shock. During this period, heart rate, PQ interval and gross activity were recorded. Subsequently, rats were returned to their home cages where heart rate, PQ interval and gross activity were recorded for another 60 min (post-treatment).

2.7.3. Experiment 3: effect of i.c.v. α-helical CRH on ACTH, noradrenaline and adrenaline during conditioned fear

On day 1, a group of 33 jugular vein cannulated rats received footshocks in a shock box according to experi-

ment 2. On day 2, at $t = -15$ min baseline blood samples were drawn under resting conditions. Then the rats were infused (from $t = -15$ min until $t = -9$ min) i.c.v. with saline ($n = 17$) or α-helical CRH ($n = 16$). At $t = 0$ min, they were picked up and moved from the home cage to the shock box. None of the animals received any shock (conditioned fear). At $t = 10$ min, the rats were returned to their home cages. Blood samples were drawn at $t = -5$, 1, 5, 15 and 35 min, using the method described by Steffens (1969), with some modifications. The samples (350–400 μl) were collected in small cups containing heparin (10 μl of 500 IU/ml) and antioxidant (10 μl of EDTA, 44 mg/ml) and centrifuged (10 min, 4000 rpm, 4°C). Plasma was separated and stored at −20°C and −80°C for ACTH and catecholamine determination, respectively. The volume of sampled blood was substituted by saline, in order to avoid volume changes. Prior to the experiments, we tested whether our procedure of blood withdrawal has any effect on plasma concentrations of ACTH, noradrenaline and adrenaline. In accordance to Steffens (1969), we showed that the procedure of blood sampling has no effect on the hormonal parameters.

2.7.4. Experiment 4: effect of i.c.v. α-helical CRH on cardiac system in atropine-methyl-nitrate-treated rats

On day 1, a group of 12 rats with implanted transmitters received footshocks in a shock box according to experiment 2. On day 2, baseline heart rate and PQ interval of rats were recorded in the home cage for 30 min under resting conditions, and then rats received saline ($n = 6$) or α-helical CRH ($n = 6$) i.c.v. Fifteen minutes after i.c.v. infusion, rats were injected s.c. with atropine methyl nitrate, and 30 s later, they were exposed to conditioned fear. During this period, heart rate and PQ interval were recorded. After testing, rats were returned to their home cages where heart rate and PQ interval were recorded for another 60 min (post treatment).

All experiments were performed during the light phase of the circadian cycle between 0900 and 1300 h. The experiments were approved by the ethical committee for animal experimentation of the Medical Faculty, Utrecht University, the Netherlands.

2.8. Statistics

Gross activity (counts/min), heart rate (beats/min) and PQ interval (ms) are presented as mean changes (±SEM) in comparison to baseline. Mean ACTH, noradrenaline and adrenaline concentrations are given in picogram per milliliter (±SEM). Baseline levels of the treatment groups were compared by a Student's two-tailed *t*-test. Differences in mean cardiac and hormonal levels between the 30-min period before and 10-min period after the introduction of conditioned fear were assessed with Student's paired *t*-test. All data were analysed by a two-factor Multivariate Analysis of Variance (MANOVA) with repeated measures, with one between-subjects factor (treatment) and one repeated measures within-subjects factor (time). The treatment factor had two levels (α-helical CRH and saline) and the time factor had 12 levels (experiment 1; $t = 5$, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min; during conditioned fear), 10 levels (experiments 2 and 4; $t = 1$, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min) or five levels (experiment 3; $t = -5$, 1, 5, 15 and 35 min). In experiment 3, an additional Student's two-tailed *t*-test was performed at $t = -5$ min to evaluate effects of α-helical CRH on basal hormonal levels. P values of < 0.05 were considered significant. Due to insufficient quality of the telemetric signal, the following data were excluded from further analysis; the PQ interval of one α-helical CRH and two saline rats (experiment 2) and the PQ interval of two α-helical CRH and two saline rats (experiment 4).

3. Results

3.1. Experiment 1

α-Helical CRH was infused in rats with implanted transmitters under resting conditions in order to exclude possible agonistic function of the CRH receptor antagonist on gross activity, heart rate and PQ interval and to examine whether endogenous CRH in the brain plays a role in the tonic regulation of the autonomic nervous system and behavioural activity.

3.1.1. Gross activity, heart rate and PQ interval

In the saline group, mean baseline gross activity levels were 0.3 ± 0.1 counts/min, baseline heart rate levels were 347 ± 3 beats/min and baseline PQ intervals were 54.9 ± 2.1 ms. In the α-helical CRH group, mean baseline gross activity levels were 0.1 ± 0.1 counts/min, baseline heart rate levels were 341 ± 3 beats/min and baseline PQ intervals were 51.8 ± 0.8 ms. Baseline levels were not significantly different between the saline and α-helical CRH group. α-Helical CRH infusion had no significant treatment, time, or interaction of treatment by time effect on gross activity, heart rate or PQ interval under resting conditions.

3.2. Experiment 2

α-Helical CRH was infused in rats with implanted transmitters before conditioned fear to study the role of the endogenous CRH system in stress-induced behavioural and autonomic responses.

3.2.1. Gross activity

Mean baseline gross activity levels were 0.3 ± 0.1 counts/min in rats prior to saline treatment and 0.2 ± 0.1 counts/min prior to α-helical CRH treatment. Baseline levels were not significantly different between the saline and α-helical CRH group. Although visual observation

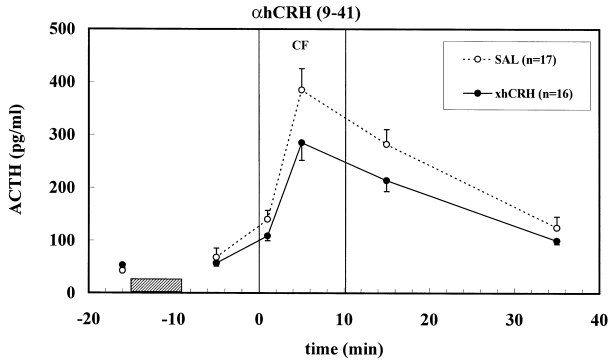

Fig. 2. ACTH levels in α-helical CRH-treated rats (xhCRH) or saline-treated rats (SAL) before, during and after 10 min conditioned fear (CF). α-Helical CRH (10 μg/3 μl) or saline was infused i.c.v. from $t = -15$ to -9 min (dark bar). Data are presented as mean ± SEM. The vertical lines mark the start and end of conditioned fear. Data that show no error bars represent average values with minimum SEM.

indicated that all rats displayed freezing behaviour during the 10-min conditioned-fear exposure, conditioned fear induced an increase in gross activity as compared to baseline home cage levels in the saline (Fig. 1A, $P < 0.001$; *t*-test) and α-helical CRH ($P < 0.01$; *t*-test) group. On average, the increase in gross activity as compared to baseline home cage levels changed significantly during conditioned fear in both groups (time effect: $F(9,216) = 5.6$, $P < 0.001$). There was a tendency for α-helical CRH treatment to reduce gross activity (treatment effect: $F(1,24) = 3.2$, $P = 0.088$).

3.2.2. Heart rate

Mean baseline heart rate levels were 377 ± 5 beats/min in rats prior to saline treatment and 374 ± 4 beats/min prior to α-helical CRH treatment. Baseline levels were not significantly different between the saline and α-helical CRH group. Conditioned fear induced a tachycardiac response in the saline (Fig. 1B, $P < 0.001$; *t*-test) and α-helical CRH ($P < 0.001$; *t*-test). On the average, the tachycardiac response elevated during conditioned fear in both groups of rats (time effect: $F(9,216) = 15.2$, $P < 0.001$). In α-helical CRH-treated rats, the conditioned-fear-induced tachycardia was significantly reduced as compared to saline-treated rats (treatment effect: $F(1,24) = 4.2$, $P < 0.05$).

3.2.3. PQ interval

Mean baseline PQ intervals were 49.2 ± 0.7 ms in rats prior to saline treatment, and 49.1 ± 0.8 ms prior to α-helical CRH treatment. Baseline levels were not significantly different between the saline and α-helical CRH group. Conditioned fear induced an increase in PQ interval in the saline (Fig. 1C, $P < 0.05$; *t*-test) and α-helical CRH ($P < 0.001$) group. In time, the PQ interval declined significantly during conditioned fear (time effect: $F(9,189) = 12.3$, $P < 0.001$). The increase in PQ interval in saline-treated rats during conditioned fear was enhanced by α-helical CRH treatment (treatment effect: $F(1,21) = 6.9$, $P < 0.05$).

3.3. Experiment 3

α-Helical CRH was infused in jugular vein cannulated rats to study the role of the endogenous CRH system in the conditioned-fear-induced ACTH and catecholamine response.

3.3.1. ACTH

In the saline group, mean baseline ACTH levels were 42 ± 5 pg/ml in rats. In the α-helical CRH group, mean baseline ACTH levels were 53 ± 8 pg/ml in rats. Baseline ($t = -15$ min) and post-infusion ($t = -5$ min) levels were not significantly different between the saline and α-helical CRH group. Conditioned fear induced an increase in ACTH levels in the saline (Fig. 2, $P < 0.001$; *t*-test) and α-helical CRH ($P < 0.001$; *t*-test) group. The increase in ACTH in saline-treated rats during conditioned fear was significantly reduced by α-helical CRH (treatment effect: $F(1,31) = 3.9$, $P < 0.05$).

3.3.2. Noradrenaline and adrenaline

In the saline group, mean baseline noradrenaline levels were 88 ± 5 pg/ml and mean baseline adrenaline levels

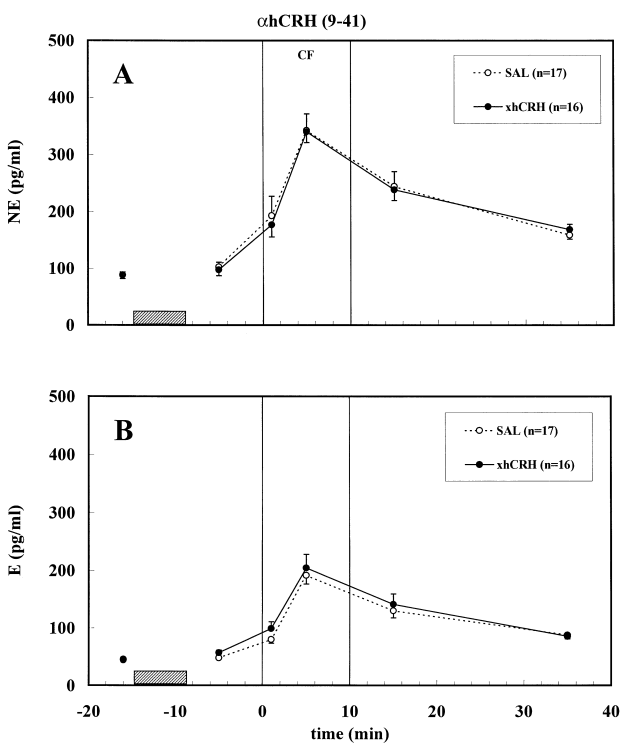

Fig. 3. Norepinephrine (NE) (A) and epinephrine (E) (B) levels in α-helical CRH-treated rats (xhCRH) or saline-treated rats (SAL) before, during and after 10 min conditioned fear (CF). α-Helical CRH (10 μg/3 μl) or saline was infused i.c.v. from $t = -15$ to -9 min (dark bar). Data are presented as mean ± SEM. The vertical lines mark the start and end of conditioned fear. Equal catecholamine levels in both treatment groups are represented as one filled circle. Data that show no error bars represent average values with minimum SEM.

were 44 ± 1 pg/ml. In the α-helical CRH group, mean baseline noradrenaline levels were 88 ± 6 pg/ml and mean baseline adrenaline levels were 46 ± 2 pg/ml in rats. Baseline ($t = -15$ min) and post-infusion ($t = -5$ min) levels were not significantly different between the saline and α-helical CRH group. Conditioned fear induced an increase in noradrenaline (Fig. 3A, $P < 0.001$; *t*-test) and adrenaline (Fig. 3A, $P < 0.001$; *t*-test) levels in the saline and α-helical CRH group. There were no significant treatment effects of α-helical CRH.

3.4. Experiment 4

Experiment 2 suggests that endogenous CRH inhibits the conditioned-fear-induced increase in vagal activity. To validate the involvement of endogenous CRH on vagal contribution during conditioned fear, rats were pre-treated with atropine methyl nitrate.

3.4.1. Heart rate

Prior to atropine injection, mean baseline heart rate levels were 333 ± 8 and 333 ± 6 beats/min in rats from the atropine/saline group and atropine/α-helical CRH group, respectively. Baseline levels were not significantly different between the groups. The changes in mean heart rate in the saline and α-helical CRH group during conditioned fear are depicted in Fig. 4A. Conditioned fear induced an increase in heart rate in the atropine/saline group ($P < 0.001$; *t*-test) and atropine/α-helical CRH group ($P < 0.001$; *t*-test). In contrast to experiment 2, no significant time or treatment effects of α-helical CRH on heart rate were found in atropine-treated rats.

3.4.2. PQ interval

Mean baseline PQ intervals were 52.6 ± 1.9 and 52.9 ± 1.2 ms in rats from the atropine/saline group and atropine/α-helical CRH group, respectively. Baseline levels were not significantly different between the groups. The changes in mean PQ interval in saline and α-helical CRH rats during conditioned fear are depicted in Fig. 4B. Conditioned fear induced a decrease in PQ in the atropine/saline group ($P < 0.001$; *t*-test) and atropine/α-helical CRH group ($P < 0.001$, *t*-test). In contrast to experiment 2, no significant time or treatment effects of α-helical CRH on PQ interval were found in atropine-treated rats.

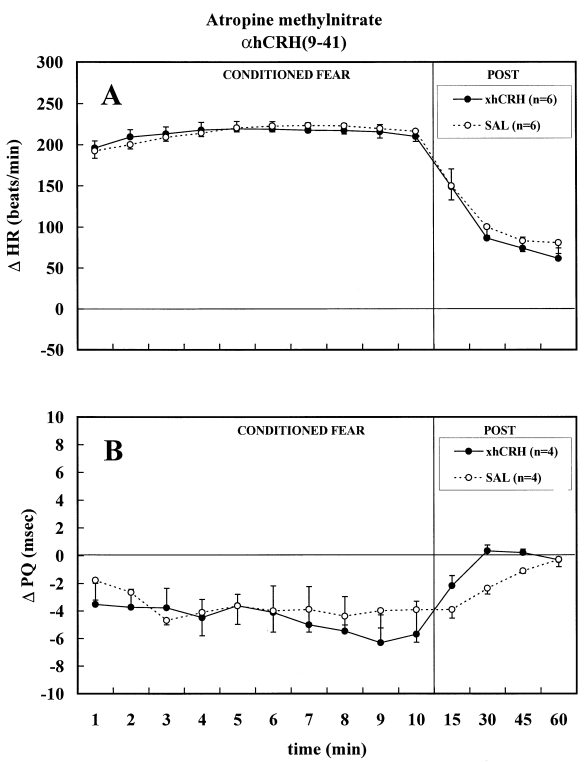

Fig. 4. Changes in heart rate (HR) (A) and PQ interval (B) in atropine methyl nitrate/α-helical CRH-treated rats (xhCRH) and atropine methyl nitrate/saline-treated rats (SAL) during and after 10 min conditioned fear. α-Helical CRH (10 μg/3 μl) or saline was infused i.c.v. from $t = -15$ to -9 min. Atropine methyl nitrate (0.3 mg/kg) was injected at $t = -30$ s. The vertical line marks the end of conditioned fear stress. Data are presented as mean ± SEM. Equal heart rate or PQ levels in both treatment groups are represented as one filled circle. Data that show no error bars represent average values with minimum SEM.

4. Discussion

We found that i.c.v. infusion of α-helical CRH in freely moving rats reduced the tachycardia, but potentiated the increase in PQ interval induced by conditioned fear. These effects were probably not mediated by inhibition of sympathetic and/or adrenomedullary activity, since the CRH receptor antagonist did not affect the stress-induced plasma noradrenaline and adrenaline responses. Moreover, the α-helical CRH-induced effects were completely blocked by pre-treatment of the rats with atropine methyl nitrate. Taken together, these results indicate that CRH, released in the brain during emotional stress, inhibits vagal outflow, thus contributing to the tachycardiac stress response.

In high doses, α-helical CRH can exhibit weak agonistic effects. It has been shown, for instance, that i.c.v. administration of 25 μg α-helical CRH induces tachycardia and behavioural activation in rats (Diamant and De Wied, 1991; Menzaghi et al., 1994). To avoid such agonistic activity, we selected a dose of 10 μg for our study, since Morimoto et al. (1993) reported that α-helical CRH in that dose does not affect baseline heart rate, blood pressure, body temperature and somatomotor activity. Indeed, we did not find any effect of i.c.v. infusion of 10 μg α-helical CRH on gross activity, heart rate, PQ interval in rats under resting conditions. Furthermore, i.c.v. infusion of 10 μg α-helical CRH did not affect plasma noradrenaline, adrenaline and ACTH levels under resting conditions before the conditioned fear exposure (at $t = -5$ min). On the other hand, i.c.v. pre-treatment with 10 μg α-helical CRH did inhibit the conditioned-fear-induced heart rate

and ACTH response. This confirms experimental evidence from studies by others and indicates that, in this dose, α-helical CRH effectively inhibits CRH signaling in the brain (Morimoto et al., 1993; Menzaghi et al., 1994; Richter and Mulvany, 1995). The absence of effects of α-helical CRH in resting rats therefore underscores the notion that endogenous CRH in the brain does not play a role in the tonic regulation of the autonomic nervous system and behavioural activity under resting, low arousal conditions (Diamant and De Wied, 1991).

Conditioned fear, i.e., forced exposure to an environment in which rats previously experienced inescapable footshocks, is a well-established model to study the impact of emotional stress in rats (Fanselow, 1980). In accordance with our previous study (Nijsen et al., 1998b), we found that the tachycardia induced by conditioned fear was associated with an increase in the concentration of plasma noradrenaline and adrenaline, as well as with an elongation of the PQ interval. The increase in PQ interval occurred almost immediately and reached its maximum well within 30 s after the start of conditioned fear, suggesting that neural rather than hormonal mechanisms underlie this effect. Since treatment of rats with atropine methyl nitrate resulted in a complete blockade of the effect, we conclude that the increase in PQ interval induced by conditioned fear is most likely to be mediated by cholinergic (vagal) activity (Nijsen et al., 1998b).

The catecholamine response indicates activation of sympathetic and adrenomedullary outflow (Lake et al., 1976). It has been shown that the tachycardia induced by various forms of emotional stress is prevented by pre-treatment of rats with a β-adrenoceptor antagonist (Kudo et al., 1983; Nakamori et al., 1993; Abdeen et al., 1995), indicating that emotional stress activates cardiac sympathetic outflow. Since sympathetic activation shortens and vagal activation lengthens the PQ interval, the conditioned-fear-induced elongation of the PQ interval must be the result of an increase in vagal activity (Nijsen et al., 1998a,b). Thus, it is concluded that activation of both the sympathetic and parasympathetic nervous systems determines the heart rate response that occurs during conditioned fear.

The aim of this study was to examine whether the endogenous CRH system is involved in the conditioned-fear-induced coactivation of both sympathetic and parasympathetic nervous systems. The i.c.v. pre-treatment of rats with α-helical CRH reduced the tachycardiac response, but potentiated the increase in PQ interval due to conditioned fear as compared to saline pre-treatment. Inhibition of sympathetic outflow likely cannot explain this effect, since α-helical CRH did not alter the sympathetic adrenomedullary response as shown indirectly by the absence of differences in plasma noradrenaline and adrenaline responses between treatment groups. The results, therefore, point to an increase in vagal outflow as the mechanism underlying the effect of the receptor antagonist on the heart rate response.

The finding, that the effect of i.c.v. infusion of α-helical CRH on the heart rate and PQ response was completely blocked by pre-treatment of the rats with the peripherally acting muscarinic receptor antagonist atropine methyl nitrate, substantiates this notion. Several points need to be considered in this respect. First, atropine treatment not only resulted in a blockade of the conditioned-fear-induced increase in PQ interval, but also led to a decrease in PQ interval as compared to baseline. In a previous study, we showed that atropine treatment under resting conditions also caused a decrease in PQ interval as compared to baseline. This suggests that the vagal system is tonically active under basal, resting conditions and explains the further decrease in PQ interval in the vagally blocked, conditioned-fear rats.

Secondly, it could be argued that atropine enhanced heart rate to a point (plateau) at which no further increase can be found after stress, thereby eliminating the possibility of observing additional sympathetic effects of α-helical CRH. We have shown previously (Nijsen et al., 1998b), however, that after pre-treatment with atropine methyl nitrate in a somewhat higher dose (0.5 mg), conditioned fear increased heart rate up to 575 beats/min, which is considerably higher than the maximal heart rate (545 beats/min) observed in the present study.

Thirdly, vagal nerves do not only exert direct cholinergic effects on effector cells of the heart, but also tonically inhibit the release of noradrenaline from cardiac sympathetic nerves (Watanabe, 1983; Levy and Martin, 1989). Thus, disinhibition of local noradrenaline release may have contributed to the effects of muscarinic blockade in our experiment. This does not affect our conclusions, since such a mechanism involves cardiac sympathetic nerves and the possible effects of α-helical CRH on sympathetic activity would therefore still be reflected in changes in heart rate and PQ interval.

Fourthly, it should be kept in mind that parasympathetic outflow to the sinoatrial and atrioventricular nodes may be differentially regulated (Furukawa et al., 1990; Wallick and Martin, 1990; Carlson et al., 1992; Gatti et al., 1995). An increase in the length of the PQ interval caused by vagal activity at the level of the atrioventricular node could thus parallel a tachycardia caused by withdrawal of vagal activity at the level of the sinoatrial node. Our present and previous results (Nijsen et al., 1998b) show, however, that pre-treatment with atropine antagonises both vagal activity at the sinoatrial (increase in conditioned-fear-induced tachycardia) and the atrioventricular nodes (blockade of the conditioned-fear-induced increase in PQ interval). This indicates that the cardiac response to conditioned fear is mediated by a simultaneous increase in vagal tone at the sinoatrial and atrioventricular nodes.

Taken together, our present data lead us to conclude that inhibition of CRH signaling in the brain with α-helical CRH counteracts the tachycardia due to emotional stress by enhancing vagal outflow. Conversely, the results indi-

cate that activation of endogenous CRH in the brain inhibits the conditioned-fear-induced net increase in vagal outflow, and thereby contributes to the tachycardiac stress response.

The present data demonstrate that 10 μg α-helical CRH reduces the emotional stress-induced ACTH response, but does not affect the stress-induced catecholamine response. This agrees with Hirasawa et al. (1991), who showed that even a much higher dose of α-helical CRH (30 μg) reduced the shaking stress-induced ACTH response without affecting the noradrenaline response in rats. These data question the role of the endogenous CRH system in stress-induced activation of the sympathetic nervous system. However, it is generally assumed that CRH acts within the central nervous system to govern sympathetic and adrenomedullary stress responses, as it has been shown that central application of CRH under resting conditions elicits increases in plasma noradrenaline and adrenaline (Brown et al., 1982; Fisher and Brown, 1991; Nijsen et al., in press), which could be blocked by α-helical CRH (Brown et al., 1985). Whatever the exact mechanisms may be, our findings indicate that the effects of exogenous administration of CRH cannot simply be extrapolated to actions of endogenous CRH released during stress.

In summary, our present data indicate that endogenous CRH, released in the brain during emotional stress, inhibits the stress-induced net increase in vagal outflow, and thereby contributes to the tachycardiac stress response.

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