JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820

Corticotropin-releasing factor receptor 1 and central heart rate regulation in mice during expression of conditioned fear

Oliver Stiedl, Michael Meyer, Olaf Jahn, Sven Ove Ögren, and Joachim Spiess

Department of Molecular Neuroendocrinology *(O.S., O.J., J.S.)*, Department of Molecular Neurobiology *(O.J., present address)*, Fractal Physiology Group *(M.M.)*, Max Planck Institute for Experimental Medicine, Göttingen, Germany

Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden *(O.S., S.O.Ö.)*

Center for Neurogenomics and Cognitive Research and Institute of Neurosciences, Vrije Universiteit Amsterdam, The Netherlands *(O.S., present address)*

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

RUNNING TITLE PAGE

Running title: Central CRF-mediated heart rate dynamics in mice

Corresponding Author:
Oliver Stiedl, Ph.D., Assist. Prof.
Center for Neurogenomics and Cognitive Research and Institute of Neurosciences, Vrije Universiteit Amsterdam, De Boelelaan 1087, Room B-256, 1081 HV Amsterdam, The Netherlands, Phone +31 20 44 47089, Fax +31 20 44 46968, E-mail: oliver@cncr.nl

Number of text pages:	34
Number of tables:	1
Number of figures:	7
Number of references:	66
Number of words in the *Abstract*:	250
Number of words in the *Introduction*:	635
Number of words in the *Discussion*:	2616

Abbreviations:

aCSF, artificial cerebrospinal fluid; AP, anterior-posterior; aSvg-30, antisauvagine-30; bpm, beats per minute; CRF, corticotropin-releasing factor; CRFBP, corticotropin-releasing factor binding protein; CRF_1, corticotropin-releasing factor receptor subtype 1; CRF_2, corticotropin-releasing factor receptor subtype 2; CS, conditioned stimulus; DPheCRF, [DPhe^{12}Nle^{21,38}]human/rat corticotropin-releasing factor $^{12\text{-}41}$; DMNV, dorsal motor nucleus of the nervus vagus; ECG, electrocardiogram; [Glu^{11,16}]Ast, [Glu^{11,16}]astressin; αhelCRF, α-helical corticotropin-releasing factor$^{9\text{-}41}$; h/rCRF, human/rat corticotropin-releasing factor; h/rCRF$^{6\text{-}33}$, corticotropin-releasing factor binding protein inhibitor; ΔHR, CS-induced heart rate increase; HR, heart rate; HRV, heart rate variability; icv, intracerebroventricular; mUcnII, mouse urocortin II; NPY, neuropeptide Y; NTS, nucleus tractus solitarius; oCRF, ovine corticotropin-releasing factor; oCRF$^{1\text{-}41}$(OH), C-terminally desamidated ovine corticotropin-releasing factor; $rCRF_1$-NT, N-terminus of rat corticotropin-releasing factor receptor 1; RMSSD, square root of the mean of the sum of successive (RR interval) differences; US, unconditioned stimulus.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Section assignment: Neuropharmacology

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The present study was performed to (i) determine heart rate (HR) effects mediated through central corticotropin-releasing factor receptor subtypes 1 (CRF_1) and 2 (CRF_2), and to (ii) investigate the contribution of endogenous CRF to baseline HR and its fear-induced adjustment in freely moving mice. CRF ligands were injected into both lateral ventricles (icv) 15 min before the presentation of a conditioned auditory fear stimulus (CS). Initial behavioral results suggest an ovine CRF (oCRF)-mediated enhanced baseline fear and mildly enhanced conditioned auditory fear. In contrast, icv injection of oCRF (35-210 ng/mouse) dose-dependently decreased baseline HR, increased HR variability, and attenuated the CS-induced tachycardia. This effect is suggested to depend on a combined activation of sympathetic and parasympathetic activity referred to as enhanced sympathovagal antagonism. An extreme bradycardia was elicited by oCRF-injection into the lower brainstem. All HR effects were probably mediated by CRF_1 as injection of the CRF_2-selective agonist mouse urocortin II was ineffective and the baseline bradycardia by icv CRF was preserved in CRF_2-deficient mice. Injection of various CRF receptor antagonists including the CRF_2-selective antisauvagine-30 did not affect the conditioned HR response. This finding suggests that endogenous CRF does not contribute to the fear-mediated tachycardia. Thus, the hypothesis of an involvement of CRF in HR responses of mice to acute aversive stimulation is rejected. Pharmacological evidence points at the involvement of CRF_1 in enhanced sympathovagal antagonism, a pathological state contributing to elevated cardiac risk, while the physiological role of the brain CRF system in cardiovascular regulation remains to be determined.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Corticotropin-releasing factor (CRF), a 41 amino acid peptide, is the principal activator of the hypothalamic-pituitary-adrenal axis in response to stressful stimulation (Vale et al., 1981). CRF is also regarded as the key molecule involved in immune, behavioral and autonomic responses to stressful stimulation (De Souza and Grigoriadis, 1995; Heinrichs et al., 1995; Owens and Nemeroff, 1991). The CRF system is hyperactive in major depression (Mitchell, 1998; Kasckow et al., 2001; Keck and Holsboer, 2001), a disorder that is associated with elevated risk for cardiovascular disease (Roose, 2001). Moreover, CRF is elevated in the cerebrospinal fluid of patients suffering from post-traumatic stress disorder (Baker et al., 1999). Among the neuroanatomical systems mediating the responses to stressful stimulation the amygdala complex is of major importance. Different nuclei of the amygdala are involved in the acquisition and expression of emotional behavioral and autonomic responses indicative of fear and anxiety (reviewed by LeDoux, 2000). For instance, in pigs subjected to coronary artery occlusion inactivation of the amygdala prevents ventricular fibrillation induced by restraint stress (Carpeggiani et al., 1992). However, in rats both appetitive and aversive events cause release of CRF in the central nucleus of the amygdala (Merali et al., 1998, 2004).

Most previous investigations addressing the role of CRF in autonomic regulation have been conducted following central and peripheral injections in rats. In rats intracerebroventricular injection of CRF elicits a profound tachycardia (reviewed by Parkes et al., 2001), that is interpreted to mimic the autonomic activation in response to stressful events. Based on such studies, CRF is regarded to be a mediator of stress responses that involve cardiovascular adjustments (as an evolutionary adaptation) to threatening environmental stimuli (Fisher, 1989, Owens and Nemeroff, 1991). However, the knowledge on the role of central CRF-mediated tachycardia in rats is mainly based on injections of unselective CRF receptor agonists (reviewed by Parkes et al., 2001) which does not allow a definition of the precise role of endogenous CRF and its receptors. Novel CRF peptides with different affinities for CRF receptor subtype 1 (CRF_1) and CRF receptor subtype 2 (CRF_2) suggest a

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

more complex functional role of the CRF system (Hsu and Hsueh, 2001; Reyes et al., 2001). Moreover, only few studies have combined autonomic and behavioral analysis (Nijsen et al., 2000). One possibility to further delineate the role of CRF in cardiovascular regulation is using genetically modified mice that do not express specific CRF receptor subtypes. In addition, the role of endogenous CRF remains to be determined by the use of subtype-selective CRF receptor antagonists.

Therefore, the objectives of the present study were to investigate the roles of central CRF_1 and CRF_2 in autonomic regulation in C57BL/6N mice and CRF_2-deficient mice. Autonomic regulation was investigated by the use of auditory fear conditioning which is an established paradigm to study conditioned HR responses in mice (Stiedl and Spiess, 1997; Stiedl et al., 1999b; Tovote et al., 2004). The presentation of an aversively conditioned auditory stimulus (CS; tone) elicits a pronounced tachycardia in freely moving mice monitored by ECG radio-telemetry. As animals are tested in their home cage, unspecific aversive stimulation is avoided and HR is at basal physiological levels. Thus, presentation of the CS in the home cage serves as an acute emotional stressor under otherwise stress-free conditions. By investigating HR effects before and during tone presentation, discrimination of the effects of CRF on baseline HR from emotional HR adjustment is possible. This procedure also allows the examination of a potential interaction between baseline HR effects and stress-induced HR adjustment by CRF. The different affinities for CRF_1, CRF_2 and the CRF binding protein (CRFBP) (Behan et al., 1996; Eckart et al., 2001) were used to select CRF_1 and CRF_2 agonists and antagonists. Additionally, CRF_1 and CRF_2 antagonists were employed to determine the contribution of endogenous CRF to fear-induced autonomic changes via central pathways.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Material and Methods

Subjects. In this study, 141 male C57BL/6NCrlBR (6N) mice (Charles River, Sulzfeld, Germany) were tested. Additionally, CRF_2-deficient (-/-) mice were derived from F1 intercross between 129SvJ and C57BL/6J mice (Kishimoto et al., 2000), and 17 male and female F3 hybrids obtained from breeding of heterozygous (+/-) mice were examined (Stiedl et al., 2003). Wildtype littermate controls (+/+) were used in comparison to CRF_2-deficient (-/-) mice. Genotyping was performed by PCR-based DNA amplification using primer sets recognizing the third intracellular loop of CRF_2 or the neomycin-resistance coding sequence as previously described (Kishimoto et al., 2000). All mice were 10-12 weeks of age at the time of testing. All mice were group housed (by the supplier or in the breeding unit) until an age of 8 weeks. Thereafter, all mice were individually housed in standard Macrolon cages (type 2: 22 x 16 x 13 cm) with free access to food and water, and maintained on a 12 hr light-dark cycle with lights switched on at 7 a.m. Single housing occurred during the duration of the experiments for several reasons. Group housing of mice results in initial fighting among individuals of newly assembled groups because they are unlikely composed of littermates. In socially unstable groups of male mice individuals may experience a chronic stress situation (Van Loo et al., 2004). Given some burden by experimental procedures, i.e. ECG transmitter implantation and cannula implantation, we tried to eliminate any additional stress potentially elicited by the establishment of new dominance hierarchy within each cage. While social isolation is a stressor in rats leading to impairment in contextual fear conditioning (e.g. Rudy, 1996), we failed to replicate this finding in group-housed male mice (littermates). Therefore, we conclude that male C57BL/6J or 6N mice would not suffer from "social isolation" to a similar degree as reported in rats (unpublished results). This conclusion is in line with a recent study suggesting a low anxiety profile in male mice housed singly up to 42 days (Bartolomucci et al., 2003). More important, we also wanted to suppress the common attitude of mice and rats in damaging (gnaw

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

away) the cannulae of cage mates. Finally, the measures of interest addressed *relative* physiological changes where, in first (and reasonable) approximation, all background effects (related to a potential isolation syndrome) effectively cancel out. Hence, single versus group housing is not expected to affect the relative changes of HR responses.

The experiments were performed during the light phase. All experimental procedures were in accordance with the European Council Directive (86/609/EEC) by permission of the Animal Protection Law enforced by the District Government of Lower Saxony, Germany.

Surgery, heart rate measurements and data evaluation. ECG transmitters (Data Sciences, TA10EA-F20, St. Paul, MN) were implanted intraperitoneally with two electrodes placed subcutaneously as previously described (Stiedl and Spiess, 1997). The mice were allowed 14-21 days to recover from surgery. The ECG was recorded telemetrically in the home cage of each mouse during the tone-dependent memory test. Memory tests were performed 24 hr after training. In the tone-dependent memory test, a 180-s pause without stimulation (pre-CS phase) was followed by a 180-s period of tone presentation (CS phase) and a subsequent 30-s period without tone (post-CS phase). The ECG signal (lead II) was digitized at a rate of 4 kHz and stored for later off-line analysis. Unrecognized beats in the ECG recording were edited and artifacts excluded from the analysis. Cardiac interbeat intervals (ms) derived from successive R waves of the ECG signal were converted into instantaneous HR (bpm). For statistical analysis, HR values were averaged over the complete pre-CS phase (180 s), while the CS phase was subdivided into six 30-s intervals. HR variability (HRV), a parameter to detect changes in the sympathovagal balance ranging from physiologic to pathologic states (Malik, 1996; Stein and Kleiger, 1999), was determined by the square root of the mean of the sum of successive RR interval differences (RMSSD). Unlike conventional statistical time-domain measures of the variability of a given time series such as variance, the analysis of RMSSD is based on time increments between consecutive beats and has been demonstrated to converge rapidly to stable values in the presence of nonlinearity and/or nonstationarity of the data stream (Meyer and Stiedl, 2003). Statistical evaluation was performed by

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

analysis of variance (ANOVA) and ANOVA for repeated measures. The post hoc comparison was performed using the Fisher´s protected least significant difference test at a significance level of $P<0.05$.

Peptides and administration. CRF and related peptides were synthesized as described previously (Rühmann et al., 1998; Jahn et al., 2001). CRF receptor agonists and antagonists (Tab. 1) including the highly potent non-selective CRF receptor antagonist [$Glu^{11,16}$]astressin ([$Glu^{11,16}$]Ast; Eckart et al., 2001), and the CRFBP inhibitor human/rat CRF^{6-33} (h/rCRF^{6-33}; Jahn et al., 2004) were initially dissolved in 10 mM acetic acid, before distilled water and two-fold concentrated sterile artificial cerebrospinal fluid (aCSF) were added to obtain the final concentration. Only α-helical CRF^{9-41} (αhelCRF) and the CRF_2-selective antagonist antisauvagine-30 (aSvg-30; Rühmann et al., 1998) were dissolved in aCSF. These solutions were adjusted to pH 7.4 and an osmolarity of 300 ± 10 mOsm. Mouse urocortin II (mUcnII), a recently discovered endogenous CRF_2-selective agonist (Hsu and Hsueh, 2001; Reyes et al., 2001), was dissolved in sterile saline at pH 5.5. By using saline as solvent, high concentrations of mUcnII could be obtained (maximum solubility c_{max} > 1500 µM) that were not soluble in aCSF at pH 7.4 (c_{max} = 5 µM) (O. Jahn and J. Spiess, unpublished observation). The maximum solubility was determined as described previously (Eckart et al., 2001). Initially, the dose-dependent effect of oCRF was tested by injection of 35, 70 and 210 ng as similar experiments in mice have not yet been performed. The dose of 210 ng (45 pmol) was selected for further tests due to its profound effect. CRF agonists in the dose range of 100-200 ng have been shown to modulate acquisition of fear conditioning in mice (Radulovic et al., 1999**).** The competitive peptidic antagonists were applied in a three-fold molar excess (150-200 pmol per mouse) over the highest agonist doses. This dose range was selected to ensure a significant antagonistic effect while preventing solubility problems which may occur with higher concentrations of peptidic CRF antagonists. Injections of aCSF and saline, when appropriate, served as control experiments. As the HR responses of aCSF-injected mice did not differ from that of

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

saline-injected mice, these data were collapsed.

Peptide solutions were injected via a 26 gauge double injector inserted into a double guide cannula system (C235, Plastics One, Roanoke, VA) during a brief (1.5-2 min) isoflurane inhalation anesthesia. The double injector was connected by medical grade polyethylene tubing to two gas-tight 25-µl syringes. These were placed in a micro-injection system (CMA/100, CMA/Microdialysis, Solna, Sweden), that delivered 0.25 µl solution at a rate of 0.33 µl/min into both hemispheres. After the end of the injection, the injector remained in place for another 15 s, in order to enhance diffusion and reduce backflow into the cannula guides, before it was replaced by the dummy cannula. Thereafter, the dust cap was screwed back onto the guide cannula. Bilateral injection sites were the lateral ventricle (icv) and the anterior proximity of the dorsal motor nucleus of the nervus vagus (DMNV) in the brainstem. The guide cannulae were implanted using a high precision stereotaxic system with 1 µm resolution (Cartesian Research, Sandy, OR) during deep anesthesia (1.2% avertin, 0.6 ml i.p./mouse, 0.024 ml/g body weight ip) 4-5 days before the injections. The bilateral injection sites were determined according to the Mouse Brain Atlas (Franklin and Paxinos, 1997) by anterior-posterior (AP) coordinates relative to the bregma position and the midsagittal suture line (icv: AP 0 mm, lateral ±1 mm, depth 3 mm; DMNV: AP -6.85 mm, lateral ±1 mm, depth 5 mm). The exact (i.e. actually delivered but not theoretically dissolved) peptide concentration of each injection solution was determined from aliquots collected after the injections, with amino acid analysis after hydrolysis with 6 M HCl in the presence of norvaline as internal standard (Eckart et al., 2001). The precise injection sites were verified after the completion of the experiments by injecting 0.25 µl methylene blue solution and histological evaluation of coronal brain sections. Additional counterstaining with nuclear fast red was used for better visualization of the target sites (Stiedl et al., 2000). Data were evaluated only from those mice that had received injections in the correct target sites.

Behavior paradigm. The fear conditioning experiments were carried out with a computer-controlled fear conditioning system (TSE, 303410, Bad Homburg, Germany) as described previously (Tovote et al., 2004). Training (acquisition) was performed in a clear acrylic plastic cage

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

(36 x 21 x 20 cm) within a constantly illuminated (~300 lx) fear conditioning box made of dark gray acrylic plastic (context 1). The ventilated fear conditioning box was thoroughly cleaned with 70% ethanol before each experiment. In the conditioning box, a loudspeaker (Conrad, KT-25-DT, Hirschau, Germany) provided constant background noise (white noise, 68 dB SPL) for 180 s followed by a 30-s tone (10 kHz, 75 dB SPL, pulsed 5 Hz) serving as the conditioned stimulus (CS). The tone was terminated by a footshock (0.7 mA, 2 s, constant current) serving as unconditioned stimulus (US). The US was delivered through a stainless steel floor grid. The mice were returned to their home cages from the fear conditioning box 30 s after shock termination. Tone-dependent memory tests were performed in a novel context (context 2) 24 hr after training to evaluate behavioral parameters that depend on exploratory activity displayed in a new environment. Context 2 was a clear acrylic plastic cage of the same size as context 1 but without surrounding box. Context 2 was cleaned with 1% acetic acid before each experiment. A small amount of home cage embedding was transferred to context 2 in order to mimic home cage-like conditions and to reduce generalized fear (Stiedl et al., 1999a). The light intensity in context 2 was adjusted to ~420 lx. Context 2 had a plain floor and the background color was white in order to render context 2 different from context 1. The behavioral performance was analyzed in a 180-s period without CS presentation (pre-CS phase) followed by 180-s period of CS presentation (CS phase). Freezing, defined as the lack of all movements besides respiration and heart beat, was visually assessed by a time-sampling procedure at 10 s intervals, while computer-controlled activity was sampled at 10 Hz sampling rate by a photobeam system and analyzed as described previously (e.g. Stiedl et al., 1999b; 2000).

In separate groups of mice HR measurements during the tone-dependent memory test were performed 24 hr after training in the home cage of each mouse to avoid all unspecific aversive stimulation and to minimize the contribution of physical activity to HR elevations. Each home cage was placed below a loudspeaker immediately after an injection. Injections were applied 15 min before the tone-dependent memory test. In case of two successive injections, CRF receptor

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

antagonists and agonists were injected 30 min and 15 min, respectively, before the tone-dependent memory test. The tone-dependent memory test using HR measurements consisted of a 180-s period without stimulation (pre-CS phase) followed by 180-s period of tone presentation (CS phase). After termination of the CS, HR was recorded for an additional 30-s period (post-CS phase). One day after the tone-dependent memory test, mice were retrained by six tone-US pairings separated by 30-s intervals (pause), before they were retested 24 hr later as described previously (Stiedl et al., 1999b). Retraining does not alter the conditioned HR response in C57BL/6N mice (Stiedl et al., 1999b).

Comparison of the heart rate effects of CRF and NPY. In order to compare the observed effects of icv-injected CRF with that recently described for icv-injected neuropeptide Y (NPY; see Tovote et al., 2004), additional experiments were performed with 210 ng oCRF and 500 ng NPY and recording of HR for 18 min in the home cage starting 15 min after icv injection. Additionally, HR responses in the tone-dependent memory tests after injection of 210 ng oCRF were compared with responses after injection of 500 ng NPY. These results originate from the study by Tovote et al. (2004; Fig. 2) with permission from Elsevier.

CRF_1 immunohistochemistry. Brains of previously anaesthetized and transcardially perfused naive mice (n = 5) were processed for immunohistochemical analysis as described previously (Radulovic et al., 1998). Coronal brain sections were incubated with a polyclonal antibody directed against the N-terminus of rat CRF_1 ($rCRF_1$-NT) that is selective for CRF_1 and does not bind to CRF_2 and CRFBP (Sydow et al., 1997). The affinity-purified antibody, anti-$rCRF_1$-NT, were used at a concentration of 0.625 µg/ml. The specificity of immunostaining was verified on brain sections that were incubated with anti-rCRF1-NT preadsorbed overnight at +4˚ C with a 10-fold excess of $rCRF_1$-NT. Isotype control sections were incubated with 0.625 µg/ml normal rabbit IgG. The coronal sections were mounted, dehydrated and coverslipped with Eukitt.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Behavioral effects of oCRF after icv injection. In a novel context (context 2), mice injected with ovine CRF (oCRF) 15 min before the memory test exhibited increased pre-CS freezing and reduced activity in comparison to aCSF-injected control mice [freezing: $F(1,21) = 24.24$, $p < 0.0001$; activity: $F(1,21) = 6.78$, $p < 0.05$; Fig. 1]. A significant CS-induced increase of freezing and decrease of activity was observed in the CS-phase when compared to the pre-CS phase. In the CS phase, mice injected with oCRF displayed significantly increased freezing indicating enhanced fear when compared with aCSF-injected mice [$F(1,21) = 4.41$, $p < 0.05$], whereas the mean activity was not significantly different [$F(1,21) = 2.35$, $p > 0.14$; Fig. 1].

HR effects of CRF receptor agonists after icv injection. *Baseline HR (pre-CS phase):* Icv injection of the CRF agonists tested 15 min before the memory test significantly affected the baseline HR of mice in the memory test (in the home cage) as indicated by ANOVA [$F(5,47) = 5.28$, $p < 0.001$; Figs. 2A, 3A]. Post hoc comparison indicated a significantly lower HR of mice injected with 70 ng ($p < 0.002$) or 210 ng oCRF ($p < 0.001$) but not with 35 ng oCRF ($p > 0.21$) than in aCSF-injected controls (Fig. 3A). Also 170 ng h/rCRF reduced baseline HR to the same degree as 210 ng oCRF ($p > 0.67$), while mUcnII ($p > 0.43$) did not significantly affect HR.

The HRV determined on the basis of the RMSSD, was significantly affected in the pre-CS phase by the injected peptide [$F(5,47) = 3.41$, $p < 0.05$; Fig. 3B]. Post hoc comparison indicated a significantly increased HRV of mice injected with 70 ng ($p < 0.005$) or 210 ng ($p < 0.01$) of oCRF and hrCRF ($p < 0.05$) when compared with aCSF-injected mice (Fig. 3B). No significant HRV effect was detectable in mice injected with 35 ng oCRF ($p > 0.23$) and mUcnII ($p > 0.54$). The effect of 170 ng h/rCRF on HRV was similar to that of 210 ng oCRF ($p > 0.72$).

In an additional experiment HR and HRV of CRF_2-deficient (-/-) mice and littermate wildtype

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

controls (+/+) were evaluated after icv injection of 270 ng h/rCRF. In both genotypes, baseline HR reflected a strong bradycardia (Fig. 3A) and HRV was extremely elevated (Fig. 3B) due to increased occurrence of sine node arrhythmias and atrio-ventricular blocks. However, both genotypes did not reveal any significant differences in HR [$F(1,15) = 0.56$, $p > 0.46$] and HRV [$F(1,15) = 0.23$, $p > 0.64$] that could be attributed to the lack of functional CRF_2.

HR during tone-presentation (CS phase): The HR patterns of the mice indicated significant differences due to the injected peptides as evaluated by analysis of variance (ANOVA) for repeated measures [$F(5,47) = 13.01$, $p < 0.0001$; Fig. 3C]. The CS-induced tachycardia (ΔHR in the first min related to pre-CS HR) during the first minute of tone presentation was significantly blunted by injection of oCRF and h/rCRF [$F(5,47) = 4.09$, $p < 0.005$; Figs. 2, 3C, 3D]. The post hoc comparison indicated a significantly lower CS-induced tachycardia after injection of 210 ng oCRF ($p < 0.001$) and 170 ng h/rCRF ($p < 0.05$) than of aCSF (Fig. 3C). The ΔHR of aCSF- and mUcnII-injected mice did not differ from one another ($p > 0.96$).

HR effects of CRF receptor antagonists after icv injection. The non-selective CRF receptor antagonists αhelCRF, [DPhe^{12}Nle^{21,38}]h/rCRF$^{12-41}$ (DPheCRF), [Glu^{11,16}]Ast and the CRF_2-selective antagonist aSvg-30 did not affect the characteristic HR pattern in the memory test when injected alone [$F(4,31) = 1.36$, $p > 0.27$; Fig. 4A]. The HR response of mice injected with aSvg-30 indicated a trend but was not significantly ($p = 0.61$) elevated during and after tone presentation in aSvg-30-injected mice when compared with HR of aCSF-injected controls. Neither the pre-CS HR [$F(4,31) = 0.89$, $p > 0.48$], nor the CS-induced HR increase [$F(4,31) = 0.67$, $p > 0.61$; mean ΔHR approximately +120 bpm] were significantly affected by the employed CRF receptor antagonists when compared with control values of aCSF-injected mice.

Injections of the CRF receptor antagonists DPheCRF and [Glu^{11,16}]Ast applied 15 min before the injection of oCRF were performed to investigate the specificity of the oCRF effect (Fig. 4B). Pre-

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

treatment with 640 ng DPheCRF significantly attenuated the oCRF-mediated effect [$F(1,15) = 13.01$, $p < 0.005$]. However, the HR pattern was significantly lower than that of aCSF-injected controls [$F(1,13) = 5.64$, $p < 0.05$]. The most effective antagonist was [Glu^{11,16}]Ast which completely blocked the effect mediated by 143 ng oCRF resulting in a HR pattern that did not differ significantly from that of aCSF-injected mice [$F(1,13) = 0.17$, $p > 0.68$].

HR effects of an inactive CRF peptide and a CRFBP inhibitor after icv injection. An additional control experiment was performed by injection of the inactive compound the C-terminally desamidated oCRF (oCRF$^{1-41}$(OH); Figs. 2A, 4B). This experiment was designed to investigate any unspecific effects on HR mediated by this CRF-related peptide as it lacks significant binding affinity to CRF_1 similarly as shown for the C-terminally desamidated analogs of h/rCRF (Behan et al, 1996) and bovine CRF (Chalmers et al., 1996). Neither pre-CS HR [$F(1,16) = 0.16$, $p > 0.69$; Fig. 4B] nor pre-CS HRV [$F(1,16) = 0.26$, $p > 0.61$; data not shown] of mice injected with oCRF$^{1-41}$(OH) differed significantly from the corresponding values of aCSF-injected controls. In addition, the HR pattern of mice injected with oCRF$^{1-41}$(OH) did not differ significantly from the HR pattern of aCSF-injected mice in response to tone exposure [$F(1,16) = 0.61$, $p > 0.44$].

Finally, an experiment was performed to test whether bound h/rCRF may be released from the CRFBP that is considered to function as a pharmacologically significant reservoir of endogenous CRF (Jahn et al., 2004). Therefore, 670 ng (209 pmol) of the CRFBP inhibitor h/rCRF$^{6-33}$ was injected. No significant effect on pre-CS HR [$F(1,11) = 2.61$, $p > 0.13$], HRV [$F(1,11) = 0.05$, $p > 0.83$] and the tone-induced HR pattern [$F(1,11) = 0.21$, $p > 0.65$] was observed when compared with the corresponding values of aCSF-injected controls (data not shown).

HR effects after brainstem injection of oCRF. Since icv injection of oCRF revealed a HR pattern that was suggestive of a predominantly parasympathetic activation, local brain injections were performed to determine whether the parasympathetic activation was mediated in specific brain areas. We selected the brainstem as we considered a dissociation of behavioral and autonomic

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

regulation downstream from the amygdala.

Injection of oCRF into the anterior vicinity of the DMNV (Fig. 5A, B) caused a dose-dependent bradycardia of the baseline (pre-CS) HR [$F(3,26) = 22.46$, $p < 0.0001$], and affected the overall HR patterns [$F(3,26) = 40.24$, $p < 0.0001$; Fig. 5C]. Post hoc comparison revealed a significantly lower pre-CS HR in mice injected with 100 ng or 210 ng oCRF compared with the pre-CS HR of aCSF-controls ($p < 0.0001$). In addition, the CS-induced tachycardia (ΔHR; HR in the first min of the CS phase related to pre-CS HR, data not shown) was significantly affected by oCRF ($F_{3,26}$=6.10, P<0.005). Mice injected with 100 ng or 210 ng oCRF responded with a significantly lower CS-induced tachycardia than mice injected with aCSF ($p < 0.005$).

Injection of the CRF receptor antagonist astressin (580 ng) 15 min before the administration of oCRF (100 ng) caused an attenuation of the oCRF-mediated pre-CS bradycardia. The pre-CS HR was significantly higher than the pre-CS HR of mice injected with 100 ng oCRF alone [$F(1,11) = 16.48$, $p > 0.005$), and was not different from that of aCSF-injected mice [$F(1,9) = 0.31$, $p > 0.59$].

Immunohistochemical analysis of CRF_1 in the mouse brainstem. Immunohistochemical analysis of CRF_1 production was performed to investigate the potential target sites for the oCRF-mediated bradycardia in the brainstem as this has not been included in a previous study (Radulovic et al., 1998). CRF_1 immunoreactivity was observed in the DMNV and - to a lesser extent - in the lateral reticular tract and the nucleus ambiguus (Fig. 6). CRF_1 immunoreactivity was weak in the nucleus tractus solitarius (data not shown).

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The results of the present study demonstrate for the first time the involvement of exogenously applied CRF in the regulation of HR in mice. The main findings are summarized as follows:

1. Icv injection of oCRF resulted in a behavioral suppression evidenced by a reduced baseline activity and increased freezing in the pre-CS phase of the tone-dependent memory test.

2. Icv injection of oCRF affected central HR regulation via CRF_1 (but not CRF_2) resulting in a baseline bradycardia and attenuated CS-induced tachycardia combined with substantially increased HRV.

3. Local injections suggest the involvement of CRF_1 in the lower brainstem for activation of the parasympathetic system.

4. All CRF receptor antagonists used in this study failed to interfere with the conditioned tachycardia elicited by the CS.

On the basis of previous reports of the CRF-mediated tachycardia in rats, an elevation of basal HR and a concomitant prolongation of the CS-induced tachycardia were hypothesized in mice. Contrary to this hypothesis, it is concluded, that CRF is not involved in the HR adjustment by conditioned fear in mice. However, exogenous CRF is a strong modulator of autonomic function and profoundly affects HR dynamics.

CRF-mediated behavioral effects in the tone-dependent memory test

The results of the behavioral experiments suggest that oCRF mediates an increase of freezing and decrease of locomotor activity in the pre-CS but not in the CS phase, i.e. behavior effects that are indicative of generalized fear. This finding is in agreement with interpretations of enhanced anxiety-like behavior by central CRF injection (Koob and Heinrichs, 1999). A substantial increase of freezing in the CS phase was hardly expected due to the relatively high freezing scores of control mice indicating a potential ceiling effect. However, centrally administered CRF failed to exhibit any

anxiety-like effects in an ethologically relevant and pharmacologically validated test that measured the latency to consume a palatable food snack (Merali et al., 2004). On the basis of the HR effects, it cannot be excluded that the behavioral suppression may be a consequence of the low cardiac output. Finally, a potential dissociation of the autonomic and behavioral responses cannot be excluded and may be attributed to differential effects of oCRF on neuronal outputs for behavioral and autonomic functions most probably downstream of the central nucleus of the amygdala.

HR effects of CRF receptor antagonists

It was tried to maintain an equimolar range for the CRF agonists used, while similar equimolar doses could not be maintained for the CRF antagonists due to substantial solubility differences. Despite the use of theoretically identical peptide amounts, primary variability of finally determined peptide doses is attributable to weighing accuracy of small amounts of lyophilized, potentially electrically charged peptides. Subsequently, the variability is increased due to unspecific binding of peptide in the microtubes after vortexing, in pipette tips during liquid transfer, and in the medical grade polyethylene tubing of the injection system. The difference between actually injected versus theoretically dissolved peptide increases with both decreasing peptide dose and increasing hydrophobicity of the peptide. Consequently, amino acid analyses were always performed to determine the actual amount rather than the theoretical peptide content delivered by the injection system. However, the variability among the different doses used is not expected to be of relevance with respect to the conclusions provided.

The maximum HR achieved in mice subjected to severe stress such as footshock exposure is approximately 800 bpm (Stiedl et al., 1999b). The CS-induced HR increase in controls transiently achieved 750 bpm, and is associated with a decreased HRV (Stiedl et al., 2003). Importantly, none of the CRF receptor antagonists used attenuated the conditioned tachycardia when injected alone. Thus, endogenous CRF probably is not involved in the CS-induced tachycardia. This seems plausible, since CRF receptor-mediated effects are relatively slow because its effects are mediated

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

via activation of the cAMP cascade. Consequently, endogenous CRF release should be effective with some delay in contrast to a fast neuronal sympathetic activation. Based on (1) the HR effects induced by low amounts of CRF, (2) the lack of effects of used CRF antagonists when given alone, (3) the prevention of CRF-induced HR changes upon pre-injection of these antagonists, and (4) the lack of HR effects of the CRFBP inhibitor, there is no other option but to conclude that CRF does not play a role in the cardiodynamic readjustment elicited by acute stress. This finding challenges the proposed role of CRF as a mediator of cardiovascular adjustments mediated by acute stress under physiological conditions. This conclusion does not rule out a role of the CRF system in cardiovascular dysfunctions due to chronic stress in pathological states.

The use of a CRF_1-selective non-peptidic antagonist is not expected to yield additional evidence besides that already provided. In fact, experiments were performed with the non-peptidic CRF_1 antagonist CP-154,526. However, the solubility of CP-154,526 in aqueous solution at neutral pH (5.5) is very low. In order to improve the solubility an acidified solution of low pH (2.5) was used as reported previously (Blank et al., 2003). Intraperitoneal injection of strongly acidified solutions both with and without CP-154,526 resulted in a long-lasting severe tachycardia that did not allow to perform proper HR measurements. Furthermore, the disadvantage of non-peptidic CRF antagonists such as CP-154,526 is that they exert their antagonistic effect by binding to a transmembrane domain rather than the binding domain for the agonists of CRF_1, suggesting an allosteric mechanism of inhibition. This mechanism may limit the receptor-specificity in view of the finding that the transmembrane domains show the highest amino acid sequence homology between the different receptor subtypes (*cf.* Eckart et al., 2002).

HR effects of CRF receptor agonists

The present results indicated that icv injection of oCRF and h/rCRF but not mUcnII results in an enhanced activation of the parasympathetic system as indicated by a relative bradycardia in the pre-CS baseline phase and a markedly blunted conditioned tachycardia. The CRF receptor agonists

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

oCRF and h/rCRF exhibit both a preference for CRF_1, however, with a significantly lower affinity of oCRF than h/rCRF to CRF_2 (Eckart et al., 2001).

The relatively low CS-induced HR increase after CRF injection resembled the HR response observed in non-conditioned mice that is interpreted to represent an attention response (Stiedl and Spiess, 1997). This result implies either an impaired recall function or an attenuated fear response. The blunted tachycardia in oCRF-injected mice appeared to be unrelated to the modulation of baseline HR by CRF. However, the substantially enhanced HRV suggests a concomitant activation of the parasympathetic and the sympathetic system. Thus, the autonomic system may have exhibited a change of basal cardiovascular state that prevented the expression of conditioned tone-dependent fear, i.e. a strong CS-induced tachycardia. An enhanced sympathetic tone may be reflected in elevated circulating epinephrine and norepinephrine after icv injection of oCRF as reported in rats (e.g. Fisher, 1989; Nijsen et al., 2000).

On the basis of differential effects of the agonists in the present study in mice, it is suggested that a CRF_1-mediated parasympathetic activation - in contrast to the previously reported CRF-mediated effects in rats - was responsible for the observed HR change (relative bradycardia). Preliminary investigations in C57BL/6J and Balb/c mice (O. Stiedl et al., unpublished observation) have unambiguously confirmed the HR results in C57BL/6N mice. Both the failure of mUcnII to mediate any HR effect and the preserved bradycardia by icv h/rCRF-injection in CRF_2-deficient mice, support our assumption that the parasympathetic activation by CRF is mediated by CRF_1. Here it is important to note that no baseline and stress-induced differences were found in HR and its variability (based on the RMSSD value) in CRF_2-deficient mice and their wildtype littermate controls (Stiedl et al., 2003). Furthermore, preliminary experiments with the newly developed CRF_1-selective peptide agonist cortagine (Tezval et al., 2004) confirmed the results on HR dynamics caused by oCRF (P. Tovote et al., unpublished observation).

Unlike the finding of a dominant bradycardia in mice in the present study, a CRF-mediated tachycardia is observed in rats that is attributed to central sympathetic activation (reviewed by

Parkes et al., 2001). In view of the finding that a prolongation of the PQ interval of the ECG was elicited by icv injection of 2 µg h/rCRF and concomitant subcutaneous administration of a β_1-adrenergic antagonist, a CRF-mediated parasympathetic activation has been suggested in addition to the predominantly sympathetic activation (Nijsen et al., 2000). Procedural differences are not expected to contribute to the observed effects of central CRF on HR responses in rat and mouse. Furthermore, the peripheral effects of CRF (reviewed by Parkes et al., 2001) do not differ in these two species (see Stiedl and Meyer, 2002; Stiedl et al., 2003). A recent study in trout also reported bradycardia and increased HRV by centrally administered CRF (Mimassi et al., 2003).

Specificity of CRF receptor-mediated effects

The experiment using oCRF$^{1-41}$(OH) confirmed that the effects of oCRF on HR were CRF receptor-mediated rather than attributable to unspecific peptidic side effects or potential effects of peptide fragments generated by endogenous peptidase activity. Moreover oCRF$^{1-41}$(OH) was used, because it binds neither to the CRFBP nor to one of the CRF receptor subtypes (O. Jahn and J. Spiess, unpublished observation).

Injections of CRF receptor antagonists prior to the application of CRF receptor agonist were performed to demonstrate the specificity of the CRF-mediated effects. The CRF receptor antagonist [Glu^{11,16}]Ast (Eckart et al., 2001) was most effective to block the oCRF-induced action on HR. Astressin was not tested on the basis of the reported failure to exhibit antagonistic action after icv injections (Brauns et al., 2001). However, astressin was effective in the inhibition of oCRF-mediated effects when injected locally in the brainstem in agreement with previous results (Radulovic et al., 1999). Consistent with the reported relatively low antagonistic potency of DPheCRF (Gulyas et al., 1995), the oCRF-mediated effect was attenuated but not fully inhibited by DPheCRF. Relatively high amounts of αhelCRF are required to antagonize CRF-mediated effects (Stenzel-Poore et al., 1994; Richter and Mulvany, 1995; Kishimoto et al., 2000). In addition, this

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

antagonist has been reported to exhibit weak agonistic effects *in vivo* (Menzaghi et al., 1994) and to act as partial agonist at CRF_1 *in vitro* (Smart et al., 1999; Eckart et al., 2001). Therefore, αhelCRF was not tested for its potency to antagonize the oCRF-mediated effect in the present study. The partially agonistic function of αhelCRF may have contributed to the relatively low pre-CS HR when injected alone. The potency of αhelCRF to release endogenous CRF from the CRFBP (Behan et al., 1995) also could have contributed to the lower pre-CS HR. However, an involvement of CRFBP in cardiovascular regulation appears unlikely as the CRFBP inhibitor h/rCRF^{6-33} injected icv failed to affect HR in mice, a finding consistent with that in the rat (Heinrichs et al., 2001). As both the CRF_2-selective antagonist aSvg-30 and agonist mUcnII did not mediate any HR effects, the contribution of central CRF_2 in HR regulation appears unlikely. Furthermore, mice lacking functional CRF_2 display intact HR responses during novelty exposure and retention of conditioned auditory fear (Stiedl et al., 2003).

Potential brain areas involved in CRF_1-mediated cardiovascular effects

Based on CRF_1 immunoreactivity (Radulovic et al., 1998), CRF_1 mRNA distribution (Van Pett et al., 2000) and central autonomic pathways (e.g. Saper, 1995; Bohus et al., 1996; Ter Horst et al., 1996) and CRF-induced FOS induction (Andreae and Herbert, 1993; Bittencourt and Sawchenko, 2000), we hypothesize that brainstem areas may be involved in the oCRF-mediated parasympathetic activation. In fact, the oCRF injection into the anterior proximity of the DMNV resulted in an extreme bradycardia generally not observed under physiologic conditions. The finding of strong CRF_1 immunoreactivity in the DMNV and relatively low CRF_1 immunoreactivity in the nucleus tractus solitarius confirms previous observations of high and low mRNA distribution encoding CRF_1 in mice in these two nuclei, respectively (Van Pett et al., 2000). A contribution of the nucleus ambiguus to the HR effects in view of its CRF_1 immunoreactivity and the described involvement in parasympathetic regulation (Mendelowitz, 1999) cannot be ruled out. Thus, the observed

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

parasympathetic enhancement by icv injection may be partially mediated by CRF_1 in these nuclei as the bradycardia after icv injection is preserved in CRF_2-deficient mice. However, due to the relatively large size of available injection systems, access to defined brain areas is limited in mice and injection may not be possible without potential lesion of the site of interest. The physiologic relevance of CRF_1 in the DMNV and the nucleus ambiguus for cardiovascular function remains to be determined. Differential CRF_1 immunoreactivity with stronger brainstem labeling and lower cortical labeling in mice than in rats has been reported (Radulovic et al., 1998). These differences may contribute to the diverging effects of central CRF on HR dynamics in these two rodent species.

Clinical relevance of heart rate dynamics and pathological implications

The beat-to-beat regulation of HR is coupled to higher autonomic control centers, peripheral feedback mechanisms, intrinsic sinoatrial node dynamics which modulate the rhythmicity of the heart's intrinsic pacemaker leading to complex fluctuations in the time series formed by consecutive cardiac interbeat intervals. While the precise origin and physiological function of the variability of HR have not been identified clearly, the measures of HR and its variability provide critical clues for the assessment of interaction of central neuropeptides and neuroautonomic cardiovascular control.

The comparison of CRF- with neuropeptide Y (NPY)-mediated cardiovascular effects reported in a previous study (Tovote et al., 2004) reveals opposite effects on the regulation of autonomic balance. The significance of HRV determined on the basis of the RMSSD measure is highlighted in the comparison of effects of oCRF and NPY on HR dynamics (Fig. 7). While the absolute HR values were similar in mice that received an icv injection of 210 ng (45 pmol) CRF or 500 ng (118 pmol) NPY, the markedly altered HRV points at the operation of substantially different mechanisms. A concomitant increase of parasympathetic and sympathetic tone results in increased HRV that is elicited in man after intravenous infusion of norepinephrine (Tulppo et al., 1998). Enhanced sympathovagal antagonism underlying the HR dynamics elicited by oCRF is associated with elevated arrhythmic risk and appears to be involved in sudden cardiac death (Zipes and

Wellens, 1998), whereas the readjustment of autonomic state induced by NPY appears to be less adverse. Consistent with this assumption, non-linear fractal analysis of HR dynamics, which addresses the scaling and intrinsic correlation properties of heartbeat interval fluctuations (reviewed by Stiedl and Meyer, 2003), did not provide evidence for a pathological state of neuroautonomic control induced by 500 ng NPY (M. Meyer et al., unpublished observation). This result is in contrast to the abnormal state of HR dynamics induced by 210 ng oCRF or the short range correlation of HR interval fluctuations due to parasympathetic inhibition (M. Meyer et al., unpublished observation). The key features of fractal analysis are based on dimensionless dynamical estimates that are essentially identical in man and mouse (Stiedl and Meyer, 2002) and help assess cardiac risk states due to neuroautonomic dysregulation in the absence of obvious ECG waveform alterations (*cf.* Meyer and Stiedl, 2003).

In conclusion, using pharmacological and genetic approaches, the present study indicates a CRF_1-mediated modulation of cardiovascular dynamics in mice with vagal activation mediated through lower brainstem nuclei. The hypothesis that CRF is involved in the physiologically occurring autonomic expression of conditioned auditory fear of the mouse is rejected. However, the concept of a CRF_1-mediated enhanced sympathovagal antagonism is proposed, which in man is considered a risk factor for the cardiovascular system. Elevated CRF levels in the cerebrospinal fluid of patients suffering from major depression and post-traumatic stress disorders (Nemeroff et al., 1984; Baker et al., 1999; Kasckow et al., 2001) may be linked to altered central autonomic regulation underlying the increased cardiac mortality observed in these patients (Roose and Spatz, 1999; Roose 2001).

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors thank Drs. Klaus Eckart, Hossein Tezval and Andreas Rühmann for peptide synthesis, Mr. Thomas Liepold for amino acid analyses, Mr. Philip Tovote for helpful comments, and Ms. Karin Birkenfeld, Sonja Kriks and Tanja Lorenz for excellent technical assistance.

REFERENCES

Andreae LC, Herbert J (1993) Expression of c-fos in restricted areas of the basal forebrain and brainstem following single or combined intraventricular infusions of vasopressin and corticotropin-releasing factor. *Neuroscience* 53:735-748.

Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, Hill KK, Bruce AB, Orth DN, Geracioti TD jr (1999) Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am J Psychiatry* 156:585-588.

Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, Parmigiani S (2003) Individual housing induces altered immunoendocrine responses to psychological stress in male mice. *Psychoneuroendocrino* 28:540-558.

Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, De Souza EB (1996) Neurobiology of corticotropin-releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. *Mol Psychiatr* 1:265-277.

Behan DP, Heinrichs SC, Troncoso JC, Liu XJ, Kawas CH, Ling N, De Souza EB (1995) Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. *Nature* 378:284-287.

Bittencourt JC, Sawchenko PE (2000) Do centrally administered neuropeptides access cognate receptors?: An analysis in the central corticotropin-releasing factor system. *J Neurosci* 20:1142-1156.

Blank T, Nijholt I, Vollstaedt S, Spiess J (2003) The corticotropin-releasing factor receptor 1 antagonist CP-154,526 reverses stress-induced learning deficits in mice. *Behav Brain Res* 138: 207-213.

Bohus B, Koolhaas JM, Korte SM, Roozendaal B, Wiersma A (1996) Forebrain pathways and their behavioural interactions with neuroendocrine regulation of cardiovascular function. *Clin Exp Pharmacol Physiol* 23:177-182.

Brauns O, Liepold T, Radulovic J, Spiess J (2001) Pharmacological and chemical properties of astressin, antisauvagine-30 and α-helCRF: significance for behavioral experiments. *Neuropharmacology* 41:507-516.

Carpeggiani C, Landisman C, Montaron M-F, Skinner JE (1992) Cryoblockade in limbic brain (amygdala) prevents or delays ventricular fibrillation after coronary artery occlusion in psychologically stressed pigs. *Circ Res* 70:600-606.

Chalmers D, Lovenberg T, Grigoriadis DE, Behan DP, De Souza EB (1996) Corticotropin-releasing factor receptors: from molecular biology to drug design. *Trends Pharmacol Sci* 17:166-172.

De Souza EB, Grigoriadis DE (1995). Corticotropin-releasing factor: physiology, pharmacology and role in central nervous system and immune disorders, in *The Psychopharmacology: The Fourth Generation of Progress* (Bloom FE and Kupfer DJ eds) pp 505-517, Raven, New York.

Eckart K, Jahn O, Radulovic J, Tezval H, van Werfen L, Spiess J (2001) A single amino acid serves as an affinity switch between the receptor and the binding protein of corticotropin-releasing factor: implications for the design of agonists and antagonists. *Proc Natl Acad Sci USA* 98:11142-11147.

Eckart K, Jahn O, Radulovic J, Radulovic M, Blank T, Stiedl O, Brauns O, Tezval H, Zeyda T, Spiess J (2002) Pharmacology and biology of corticotropin-releasing factor (CRF) receptors *Receptor Channel* 8:163-177

Fisher LA (1989) Corticotropin-releasing factor: endocrine and autonomic integration in response to stress. *Trends Pharmacol Sci* 10:189-193.

Franklin KBJ, Paxinos G (1997) *The mouse brain in stereotaxic coordinates*. Academic Press, San Diego.

Gulyas J, Rivier C, Perrin M, Koeber SC, Sutton S, Corrigan A, Lahrichi SL, Craig AG, Vale W, Rivier J (1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc Natl Acad Sci USA* 92:10575-10579.

Jahn O, Radulovic J, Stiedl O, Tezval H, Eckart K, Spiess J (2004) Corticotropin-releasing factor binding protein - a ligand trap? *Mini Rev Med Chem* (in press).

Heinrichs SC, Menzaghi F, Merlo Pich E, Britton KT, Koob GF (1995) The role of CRF in behavioral aspects of stress. *Ann NY Acad Sci* 771:92-105.

Heinrichs SC, Li DL, Iyengar S (2001) Corticotropin-releasing factor (CRF) or CRF binding-protein ligand inhibitor administration suppresses food intake in mice and elevates body temperature in rats. *Brain Res* 900:177-185.

Hsu YH, Hsueh AJW (2001) Human stresscopin and stresscopin-related peptides are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nature Med* 7:605-611.

Jahn O, Eckart K, Sydow S, Hofmann BA, Spiess J (2001) Pharmacological characterization of recombinant rat corticotropin releasing factor binding protein using different sauvagine analogs. *Peptides* 22:47-56.

Kasckow JW, Baker D, Geracioti TD jr (2001) Corticotropin-releasing hormone in depression and post-traumatic stress disorder. *Peptides* 22:845-851.

Keck ME, Holsboer F (2001) Hyperactivity of CRH neuronal circuits as a target for therapeutic interventions in affective disorders. *Peptides* 22:835-844.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MR, Spiess J (2000) Deletion of Crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. *Nature Genet* 24:415-419.

Koob GF, Heinrichs SC (1999) A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res* 848:141-152.

LeDoux, JE (2000) Emotion circuits in the brain. *Annu Rev Neurosci* 23:155-184.

Malik M (1996) Heart rate variability, standards of measurement, physiological interpretation, and clinical use. *Circulation* 93:1043-1065.

Mendelowitz D (1999) Advances in parasympathetic control of heart rate and cardiac function. *News Physiol Sci* 14:155-161.

Menzaghi F, Howard RL, Heinrichs SC, Vale W, Rivier J, Koob GF (1994) Characterization of a novel and potent corticotropin-releasing factor antagonist in rats. *J Pharmacol Exp Ther* 269:564-572.

Merali Z, McIntosh J, Kent P, Michaud D, Anisman H (1998) Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. *J Neurosci* 18:4758-4766.

Merali Z, Khan S, Michaud DS, Shippy SA, Anisman H (2004) Does amygdaloid corticotropin-releasing hormone (CRH) mediate anxiolytic-like behaviors? Dissociation of anxiogenix effects and CRH release. *Eur J Neurosci* 20:229-239.

Meyer M, Stiedl O (2003) Self-affine fractal variability of human heartbeat interval dynamics in health and disease. *Eur J Appl Physiol* 90:305-316.

Mimassi N, Lancien F., Mabin D., Delarue C., Conlon JM, Le Mével JC (2003) Induction of bradycardia in trout by centrally administered corticotropin-releasing hormone (CRH). *Brain Res* 982:211-218.

Mitchell AJ (1998) The role of corticotropin releasing factor in depressive illness: A critical review. *Neurosci Biobehav Rev* 22:635-651.

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts DC, Loosen PT, Vale WW (1984) Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. *Science* 226:1342-1344.

Nijsen MJMA, Croiset G, Stam R, Bruijnzeel A, Diamant M, de Wied D, Wiegant, VM (2000) The role of CRH type 1 receptor in autonomic responses to corticotropin-releasing hormone in the rat. *Neuropsychopharmacology* 22:388-399.

Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev* 43:425-473.

Parkes DG, Weisinger RS, May CN (2001) Cardiovascular actions of CRH and urocortin: an update. *Peptides* 22:821-827.

Radulovic J, Rühmann A, Liepold T, Spiess J (1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. *J Neurosci* 19:5016-5025.

Radulovic J, Sydow S, Spiess J (1998) Characterization of native corticotropin releasing factor receptor type 1 (CRFR1) in the rat and mouse central nervous system. *J Neurosci Res* 54:507-521.

Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE (2001) Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA* 98:2843-2848.

Richter RM, Mulvany MJ (1995) Comparison of hCRF and oCRF effects on cardiovascular responses after central, peripheral and in vitro application. *Peptides* 5:843-849.

Roose SP (2001) Depression, anxiety, and the cardiovascular system: the psychiatrist's perspective. *J Clin Psychiatry* 62:19-23.

Roose SP, Spatz E (1999) Treatment of depression in patients with heart disease. *J Clin Psychiatry* 60:34-37.

Rudy JW (1996) Postconditioning isolation disrupts contextual conditioning: an experimental analysis. *Behav Neurosci* 110:238-246.

Rühmann A, Bonk I, Lin CR, Rosenfeld MG, Spiess J (1998) Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2 □-selective antisauvagine-30. *Proc Natl Acad Sci USA* 95:15264-15269.

Saper CB (1995) Central autonomic system, in The Rat Nervous System (Paxinos G ed) pp 107-135, Academic Press, San Diego.

Smart D, Coppell A, Rossant C, Hall M, McKnight AT (1999) Characterisation using microphysiometry of CRF receptor pharmacology. *Eur J Pharmacol* 379:229-235.

Stein PK, Kleiger RE (1999) Insights from the study of heart rate variability. Annu Rev Med 50:249-261.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale W (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J Neurosci* 14:2579-2584.

Stiedl O, Spiess J (1997) Effect of tone-dependent fear conditioning on heart rate and behavior of C57BL/6N mice. Behav Neurosci 111:703-711.

Stiedl O, Palve M, Radulovic J, Birkenfeld K, Spiess J (1999a) Differential impairment of auditory and contextual fear conditioning by protein synthesis inhibition in C57BL/6N mice. *Behav Neurosci* 113:496-506.

Stiedl O, Radulovic J, Lohmann R, Birkenfeld K, Palve M, Kammermeier J, Sananbenesi F, Spiess J (1999b) Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice. *Behav Brain Res* 104:1-12.

Stiedl O, Birkenfeld K, Palve M, Spiess J (2000) Impairment of conditioned contextual fear of C57BL/6J mice by intracerebral injections of the NMDA receptor antagonist APV. *Behav Brain Res* 116:157-168.

Stiedl O, Meyer M (2003) Fractal dynamics of heart beat interval fluctuations in corticotropin-releasing factor receptor 2 subtype deficient mice. *Int Physiol Behav Sci* 37:311-345.

Stiedl O, Meyer M, Kishimoto T, Rosenfeld MG, Spiess J (2003) Stress-mediated heart rate dynamics after deletion of the gene encoding corticotropin-releasing factor receptor 2. *Eur J Neurosci* 17:2231-2235.

Sydow S, Radulovic J, Dautzenberg FM, Spiess J (1997) Structure-function relationship of different domains of the rat corticotropin-releasing factor receptor. *Mol Brain Res* 52:182-193.

Ter Horst GJ, Hautvast RWM, De Jongste MJL, Korf J (1996) Neuroanatomy of cardiac activity-regulating circuitry: a transneuronal retrograde viral labelling study in the rat. *Eur J Neurosci* 8:2029-2041.

Tezval H, Jahn O, Todorovic C, Sasse A, Eckart K, Spiess J (2004) Cortagine, a specific agonist of corticotropin-releasing factor receptor subtype 1, is anxiogenic and antidepressive in the mouse model. *Proc Natl Acad Sci USA* 101:9468-9473.

Tovote P, Meyer M, Beck-Sickinger AG, von Hörsten S, Ögren SO, Spiess J, Stiedl O (2004) Central NPY receptor-mediated alterations of heart rate dynamics in mice during expression of fear conditioned to an auditory cue. *Regul Pept* 120:205-214.

Tulppo MP, Mäkikallio TM, Seppänen T, Airaksinen JKE, Huikuri HV (1998) Heart rate dynamics during accentuated sympathovagal interaction. *Am J Physiol* 274:H810-H816.

Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue hypothalamic peptide that stimulates secretion of corticotropin and □-endorphin. *Science* 213:1394-1397.

Van Loo PLP, Van Der Meer E, Kruitwagen CLJJ, Koolhaas JM, Van Zutphen LFM, Baumans V (2004) long-term effects of husbandry procedures on stress-related parameters in male mice of two strains. *Lab Anim-UK* 38:169-177.

Van Pett K, Viau V, Bittencourt JC, Chan RKW, Li H-Y, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 428:191-212.

Zipes DP, Wellens HJJ (1998) Sudden cardiac death. *Circulation* 98:2334-2351.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Sources of financial support: This study has been supported by the Max Planck Society (J.S., M.M., O.J., O.S.), Karolinska Institutets fonder (O.S., S.O.Ö.) and the Wenner-Gren Foundations (O.S.) and the Vrije Universiteit (OS).

Address correspondence to: Dr. Oliver Stiedl, Department of Developmental and Behavioural Neurobiology, Faculty of Earth and Life Sciences (FALW), Vrije Universiteit Amsterdam, De Boelelaan 1087, Room B-256, 1081 HV Amsterdam, The Netherlands. E-mail: oliver@cncr.nl

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Fig. 1. Freezing and activity of mice in the tone-dependent memory test in context 2 one day after training. Mice received an icv injection of 210 ng (45 pmol) oCRF or aCSF (control) 15 min before the memory test. Significant differences between corresponding values of aCSF- and CRF-injected mice are indicated by asterisks: *$p < 0.05$; ***$p < 0.001$; values represent means ± SEM; aCSF, artificial cerebrospinal fluid; icv, intracerebroventricular.

Fig. 2. Typical HR patterns 15 min after icv injection of aCSF, oCRF and oCRF$^{1-41}$(OH) (A). Higher temporal resolution of the instantaneous pre-CS HR in mice injected with aCSF and oCRF (B).

Fig 3. Effect of icv injection of aCSF, oCRF, h/rCRF and mUcnII on pre-CS HR (A), pre-CS HR variability (RMSSD; B), HR patterns in the tone-dependent memory test (C) and the heart rate increase induced by the conditioned stimulus (tone; D). RMSSD, square root of the mean of the sum of successive RR interval differences; -/-, CRF_2-deficient mice; +/+, wildtype littermate controls; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Fig. 4. Effects of icv injection of CRF receptor antagonists ([Glu^{11,16}]Ast, αhelCRF, DPheCRF and aSvg-30 (A), and combined injection of CRF receptor antagonists and oCRF (B) on the HR pattern in the tone-dependent memory test. HR patterns of mice injected with aCSF and oCRF are depicted as reference.

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5. Brainstem injection site indicated on the right hemisphere of a schematic coronal brain section according to the Mouse Brain Atlas (Franklin and Paxinos, 1997) (A). Representative photomicrographs of a coronal brain section with bilateral methylene blue injection before and after counterstaining with nuclear fast red (B). Mean effects of brainstem injection of aCSF, oCRF and astressin on the HR pattern in the tone-dependent memory test (C). 4V, 4th ventricle; Amb, nucleus ambiguus; DMNV, dorsal motor nucleus of nervus vagus; NTS, nucleus tractus solitarius.

Fig. 6. CRF_1 immunoreactivity in a coronal brainstem section (A). Nuclei are labeled in the cresyl violet section (B). Strong immunoreactivity was observed in the dorsal motor nucleus of the nervus vagus (DMNV), in the nucleus of the nervus hypoglossus (Hyp; C, E), and in the lateral reticular tract (LRt; D). The nucleus ambiguus (Amb) was moderately labeled (D) and no clear labeling was found in the adjacent nuclei of the solitary tract (NTS) (C). Antibody specificity is demonstrated in the cerebellum (F) by the Purkinje cell layer (PC) labeled exclusively by the anti-$rCRF_1$-NT antibody (CRF_1 AB). CRF_1 immunoreactivity was blocked by preadsorption of the N-terminus of $rCRF_1$ ($rCRF_1$-NT + CRF_1 AB) and was not present in sections incubated with 0.625 µg/ml normal rabbit IgG (IgG). CC, central canal.

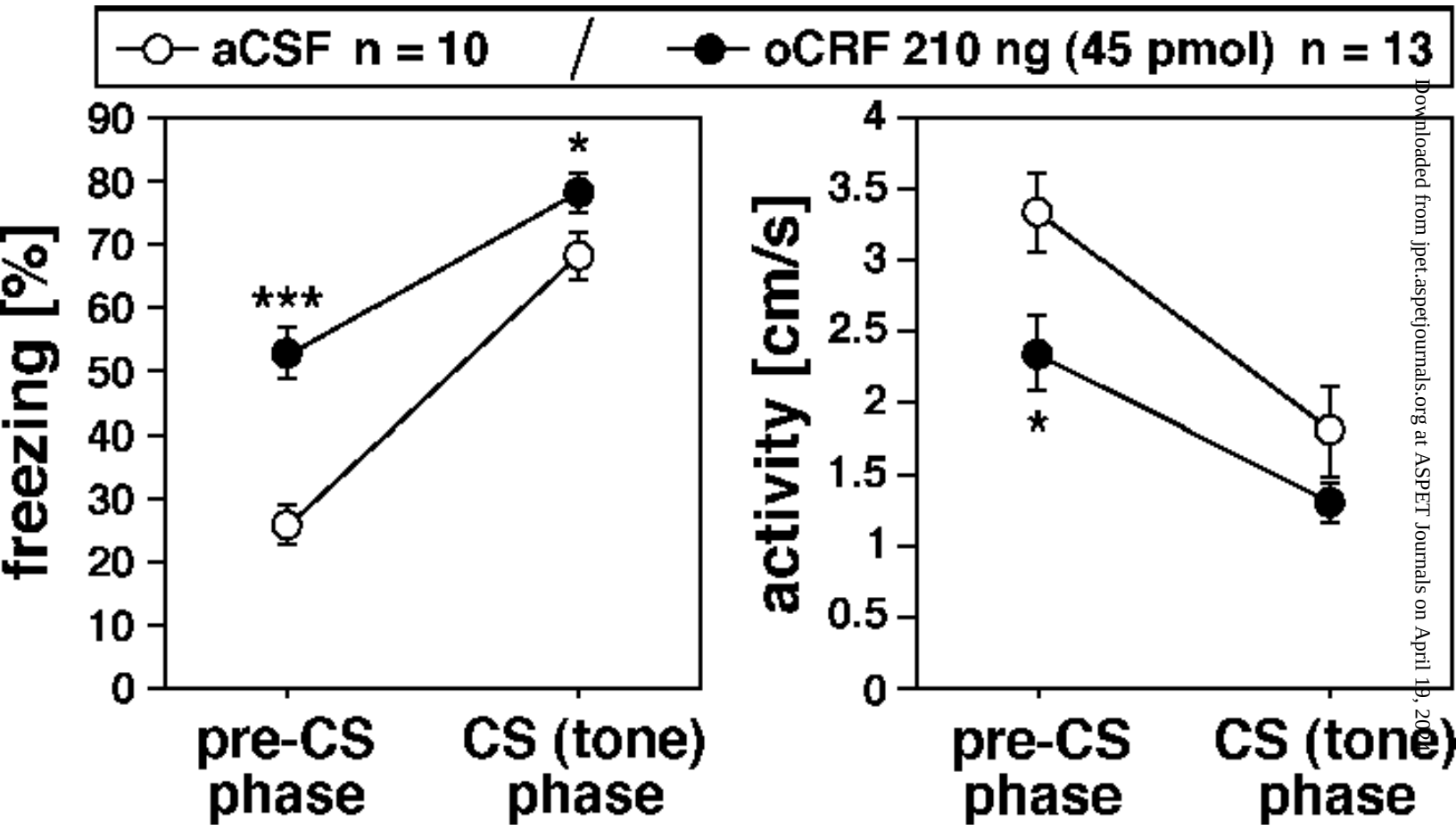
Fig. 7. Typical HR patterns in the tone-dependent memory test 15 min after intracerebroventricular injection of 210 ng (45 pmol) CRF or 500 ng (118 pmol) neuropeptide Y (NPY; B). Mean HR and HRV values of CRF- and NPY-injected mice are superimposed to indicate similar HR values ($p > 0.48$) despite substantially different HRV ($p < 0.0001$; C). NPY effects depicted in C are reproduced from Tovote et al. (2004, Fig. 2) with permission from Elsevier.

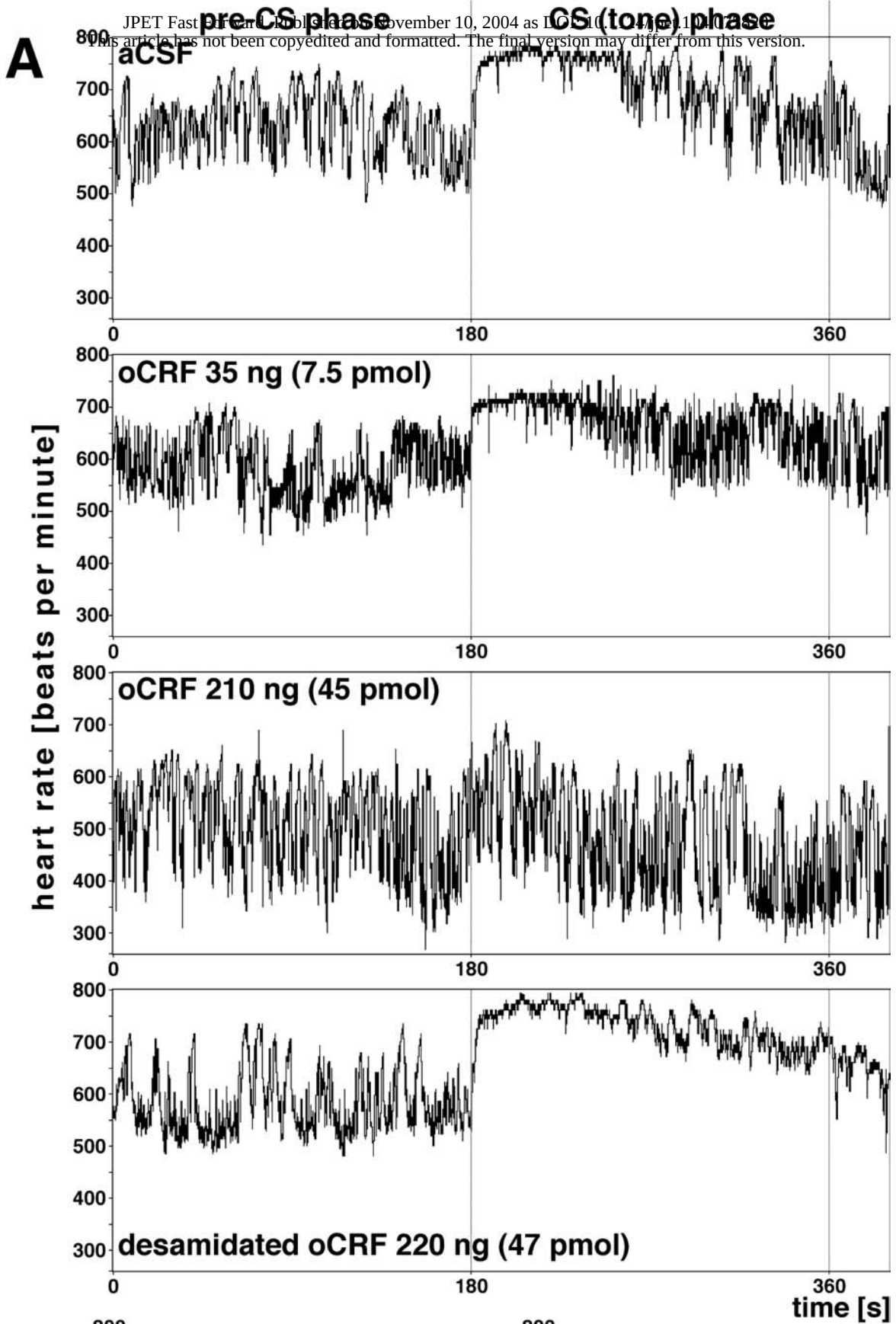
JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

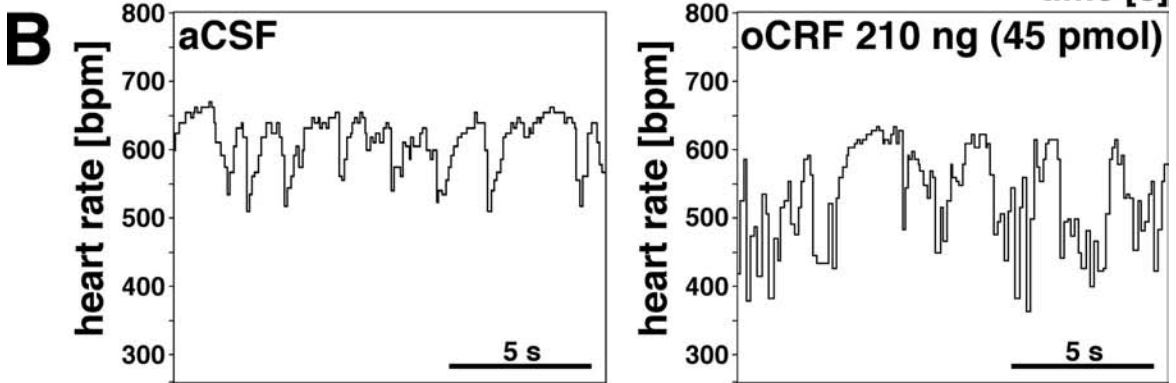
Table 1. Intracerebroventricularly injected peptides, used abbreviations, injected doses and molecular weights (MW).

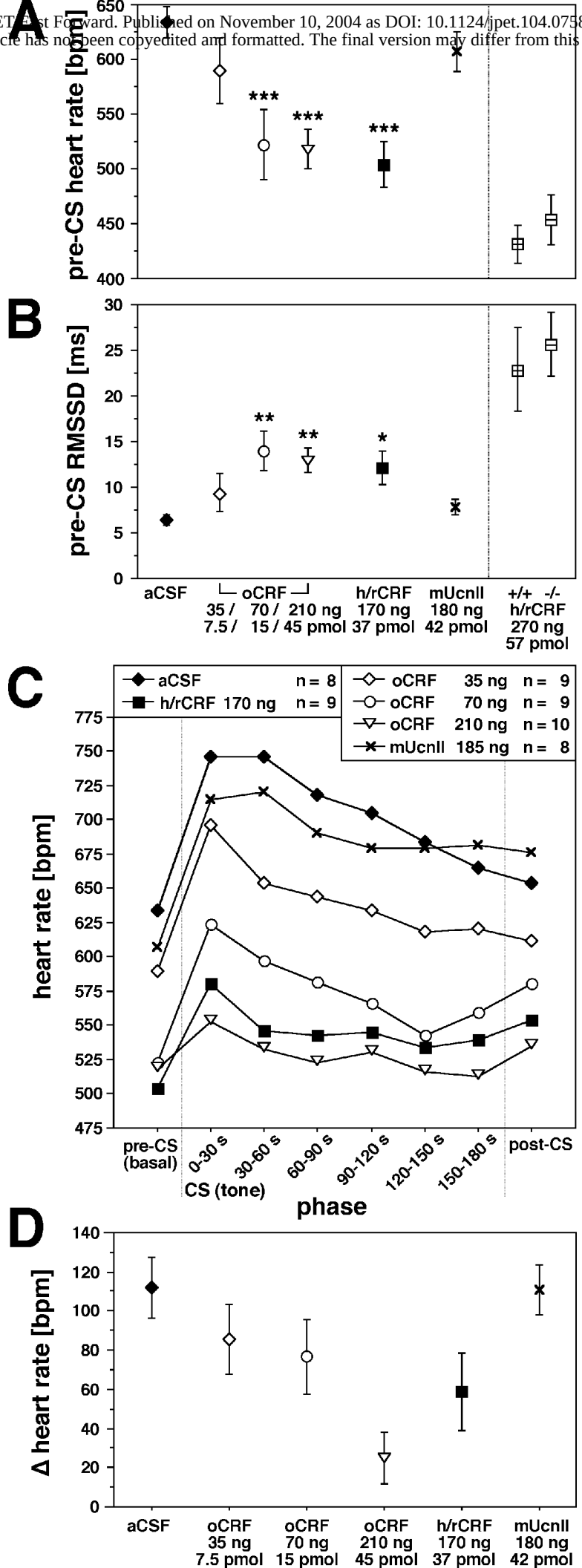
Compound	Abbreviation	Dose / mouse		MW
		ng	pmol	
CRF receptor agonists:				
ovine CRF	oCRF	35 - 210	7.5 - 45	4670
human/rat CRF	h/rCRF	170, 270	35.7, 56.7	4758
mouse urocortin II	mUcnII	180	42.2	4266
CRF receptor antagonists:				
α-helical CRF^{9-41}	αhelCRF	650	170	3826
[$DPhe^{12}Nle^{21,38}$]h/$rCRF^{12-41}$	DPheCRF	640 - 720	181 - 203	3539
[$Glu^{11,16}$]astressin	[$Glu^{11,16}$]Ast	390	107	3637
antisauvagine-30	aSvg-30	610	167	3650
CRF binding protein inhibitor:				
human/rat CRF^{6-33}	h/$rCRF^{6-33}$	670	209	3221
Inactive CRF peptide:				
desamidated oCRF	$oCRF^{1-41}$-(OH)	220	47	4671

JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.

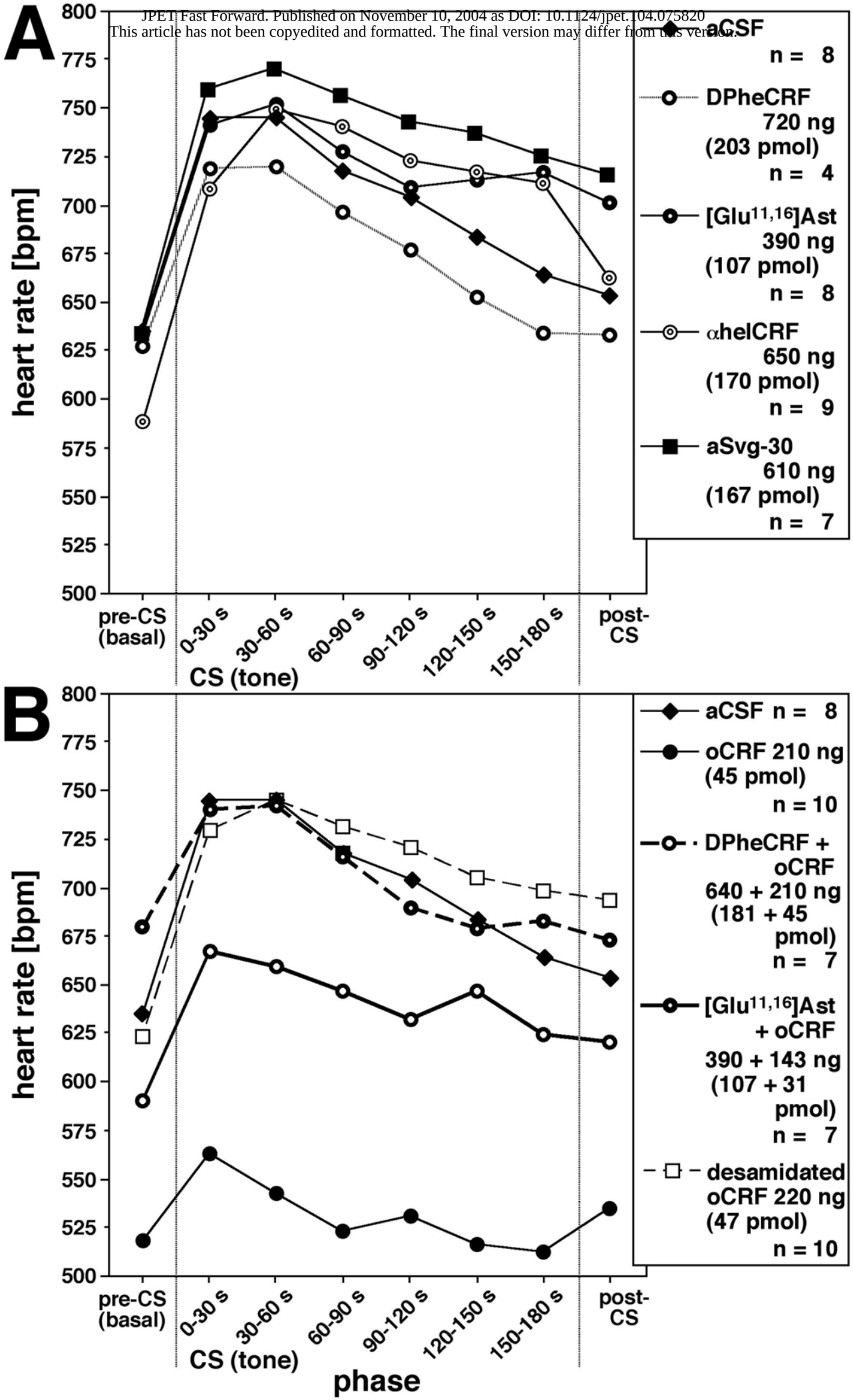








JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820



JPET Fast Forward. Published on November 10, 2004 as DOI: 10.1124/jpet.104.075820
This article has not been copyedited and formatted. The final version may differ from this version.