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Activation of central CRF receptor 1 by cortagine results in enhanced passive coping with a naturalistic threat in mice

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Summary

CRF receptor subtype 1 (CRF1), abundantly expressed in the central nervous system, has been implicated in defensive behavior in rodents. Pharmacological activation of CRF1 by peptidic agonists results in enhancement of anxiety-like behavior. However, receptor specificity of commonly used agonists was confounded by significant affinity to other receptors and widely used laboratory tests of experimental anxiety suffer from artificial aversive stimulation (e.g. electric shock), and limited measures of anxiety-like behavior. We used the recently developed, CRF1-selective agonist cortagine in a mouse model of defensive behaviors under semi-natural conditions, the Rat Exposure Test (RET). Cortagine was injected bilaterally into the cerebral ventricles (i.c.v.) of male C57Bl/6J mice, 20 min before exposure to a rat in specifically designed box that evokes a wide variety of defensive behaviors such as active/passive avoidance, freezing, risk assessment, and burying. Pre-injection of the CRF receptor antagonist acidic astressin was used to test for receptor specificity of the observed cortagine effects. A control experiment with no rat present was performed to test for baseline effects of cortagine in the exposure setup. Cortagine dose-dependently enhanced passive avoidance and freezing while burying was decreased. CRF receptor antagonism reliably blocked the effects of cortagine. Our results confirm previous findings of anxiogenic-like effects of cortagine, and demonstrate the usefulness of the RET in investigating differential patterning of drug-induced anxiety-like behavior in mice. In conclusion, our results suggest that CRF1 activation in forebrain areas promotes passive coping with the natural threat presented in the RET.

Keywords

corticotropin-releasing factor; Rat exposure test; CRF receptor 1; defensive behavior; CRF1 agonist; anxiety; active/passive avoidance; avoidance; coping

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Introduction

The 41 amino acid neuropeptide corticotropin-releasing factor (CRF) activates the hypothalamus-pituitary-adrenal (HPA) axis thereby initiating the endocrine stress response (Vale et al., 1981). Since its first characterization (Spiess et al., 1981) CRF has been demonstrated to mediate behavioral and autonomic adaptations to acute and chronic stress, and has been implicated in mediating fear responses and anxiety-like behavior across mammalian species (Bale and Vale, 2004). It exerts its effects through two known receptor subtypes, CRF1 and CRF2, with CRF1 predominantly expressed in the central nervous system (CNS). Experiments using central injections of peptidic CRF1 agonists strongly suggest a role for CRF1 in mediating anxiety-like responses to various types of aversive stimuli (Eckart et al., 1999). Previous pharmacological work on the CRF system demonstrated several site-specific roles of CRF1 in enhancing anxiety-like and defensive behaviors: CRF1 in the basolateral amygdala mediated anxiogenic-like effects in a social interaction paradigm (Spiga et al., 2005), and promoted anorexia and stress-like behaviors (Jochman et al., 2005; Bakshi et al., 2007). Increased anxiety-like behavior in the EPM was observed after activation of CRF1 in the ventral hippocampus (Pentkowski et al., 2009), or in the bed nucleus of the stria terminalis (Sahuque et al., 2006). Various peptidic and non-peptidic antagonists for CRF1 have been demonstrated to act anxiolytically (Steckler and Dautzenberg, 2006). Reduced anxiety-like behavior was reported in mice constitutively lacking the gene coding for CRF1 (Timpl et al., 1998), or in conditional knockout (KO) mice with CRF1 deficiency in limbic brain structures (Muller et al., 2003). The recently developed peptidic CRF1-selective agonist cortagine has been demonstrated to exhibit anxiogenic-like effects in the EPM, yet anti-depressive-like effects in the forced swim test (Tezval et al., 2004; Todorovic et al., 2005). Central pharmacological stimulation of CRF1 by either human/rat CRF, ovine CRF (oCRF) or cortagine has been demonstrated to strongly interfere with baseline and fear-induced autonomic activity by enhancing sympatho-vagal antagonism, which in turn results in bradycardia and increased heart rate variability (Farrokhi et al., 2007; Stiedl et al., 2005). In addition, intracerebroventricularly (i.c.v.) injected cortagine resulted in sleep disruption and freezing behavior in the homecage of mice (Farrokhi et al., 2007). When injected site-specifically into the periaqueductal gray (PAG), cortagine exerts anxiogenic-like effects in the rat exposure test (RET), a mouse model of defensive behavior under semi-natural conditions (Litvin et al., 2007).

The present study aims at investigating CRF1-mediated defensive behavior by performing intracerebroventricular injections of the CRF1-selective agonist cortagine, and the CRF1/CRF2 antagonist acidic astressin, which has improved aqueous solubility over astressin (Eckart et al., 2001). In addition, because of its somewhat unexpected pharmacological effects (Tezval et al., 2004), we aimed at characterizing cortagine in an unconventional model of mouse defensive behaviors under semi-natural conditions, the RET. In the RET, a mouse is exposed to a rat that presents a natural predator stimulus. The distinct setup of the exposure box that is divided into four different compartments, three of which are freely accessible to the mouse test subject, evokes a wide range of defensive behaviors, which can be interpreted to indicate fear and/or anxiety (Yang et al., 2004). Generally, while anxiety is a response to ambiguous aversive stimuli, fear responses are typically elicited by clearly defined threats (Blanchard and Blanchard, 2008). The RET induces both fear and anxiety-like behaviors, due to its unique setup that enables the mouse to choose its distance to the threat presented by the potential predator rat and provides opportunities to actively avoid the immediate danger. Originally, the RET was developed to focus on risk assessment behavior, which has been shown to be sensitive to several classes of anxiolytic drugs (Blanchard et al., 1990; Blanchard et al., 1993a; Blanchard et al., 1993b; Griebel et al., 1995a; Griebel et al., 1995b) and therefore presents predictive validity as a model for anxiety-like behavior. Subsequent studies revealed its power to detect differential patterns of defensive behavior after CRF receptor activation by oCRF in the PAG (Carvalho-Netto et al., 2007), and its usefulness to characterize the hypothalamic defense

system in the mouse (Martinez et al., 2008). Similar to the shock-prod test, in which rodents react to an electrical shock given by a metal probe with a variety of behaviors including avoidance, immobility and defensive burying (De Boer and Koolhaas, 2003), the RET allows for classification of active vs. passive defensive behaviors. We hypothesized that under the semi-natural conditions of the RET, pharmacological interference with CRF1 would result in a differential pattern of defensive behavior that could help to further clarify the role of central CRF1 in fear and anxiety.

Methods

Animals

Subjects were male, 8 week-old C57BL/6J mice, obtained from Jackson Laboratories. Four adult male Long-Evans rats served as natural predatory stimuli. All experimental procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Hawaii.

Experimental setup

The rat exposure box consisted of a large box (length: 562 mm, height: 223 mm, depth: 257 mm), and a smaller box (length: 138 mm, height: 82 mm, depth: 84 mm) made of gray PVC, interconnected by a clear circular plexiglas tube (inner diameter: 60 mm, length: 130 mm). The boxes were equipped with clear plexiglas walls on one side to allow observation. The large box was divided by a wire mesh (hole size: 8×8 mm) into two identically sized rectangular spaces (Fig. 1). The rat was placed behind the wire mesh on the closed side of the large box, and the mouse had free access to the opposite side of the large box (surface), tunnel, and small box (chamber).

Habituation and exposure

To reduce novelty-induced anxiety, mice were habituated to the experimental setup in two 15 min sessions on consecutive days (day 1 and 2). Forty ml of the mouse's homecage bedding (corn chops) were evenly distributed on the surface area, and 70 ml was poured into the chamber before the mouse was introduced to the middle of the surface area. No rat was present during the habituation sessions. After completion of the habituation session, mouse subjects and bedding were transferred back into the mouse's individual homecage. The setup was cleaned with 70% ethanol between mouse subjects. On the test day (day 3), a single rat was placed into the closed compartment of the large chamber. Similar to the habituation sessions, bedding material was distributed on the surface and in the chamber, before individual mice were introduced on to the surface. During the 10 min test session the behavior of mice was videorecorded for later offline analysis. The mouse compartments of the test setup were cleaned with 70% ethanol between subjects.

Intracerebral cannulation

Mice were anesthetized with 336 mg/kg Avertin (tribromoethanol), injected intraperitoneally, and fixed in a stereotaxic apparatus (Kopf series 1900, Tujunga, CA). The skull surface was exposed and cleaned with a razorblade and 70% ethanol, before holes (0.5 mm) were drilled 1 mm bilateral to bregma. Double guide cannulae, projecting 2 mm below the skull surface with dummy and dust cap (Plastics one, Roanoke, VA) were perpendicularly inserted and fixed with carboxylate dental cement (3M, St. Paul, MN).

Drugs and administration

In order to keep the stimulus rats uniformly active during and across test sessions, they were systemically injected with 5.0 mg/kg of d-amphetamine 20 (Sigma-Aldrich, St. Louis, MO)

minutes prior to testing. A new rat was used after every five trials or if cessation of movement or stereotypy was observed. Cortagine, synthesized and checked by amino acid analysis in our laboratory was dissolved in artificial cerebrospinal fluid (aCSF) as vehicle. On test day 3, mice were injected with either vehicle, CRF1 agonist cortagine, CRF receptor antagonist acidic astressin, or both, under brief isoflurane anesthesia (1 min). A volume of 0.25 μ l was injected bilaterally using two 25 μ l Hamilton syringes mounted on a microinfusion pump (CMA microdialysis, series 100, Solna, Sweden). Mice were returned to their homecage and left undisturbed for 20 min before testing. In the double-injection experiment, cortagine was applied 15 min after pre-injection of acidic astressin, and testing started after another 15 min. After completion of the experiments, mice were injected i.c.v. with methylene blue control dye, and brains were quickly extracted to be frozen over liquid nitrogen. Coronal sections were cut at 25 μ m, and correct cannula placement was verified microscopically (Zeiss Axiovert, Goettingen, Germany).

Behavior analysis and statistical evaluation

Mouse behavior and location during the RET was scored by an observer blind to the experimental conditions using custom-made scoring software (Hindsight, developed by Scott Weiss). Line crossings were calculated as the sum of transitions from surface to chamber compartments. Typical behaviors observed and scored in the RET included freezing, stretch-attend, burying/tunnel blocking, contact with and climbing on the mesh separating mouse and rat compartments. Those behaviors were analyzed according to their duration and frequency of occurrence. To analyze dose-response characteristics of cortagine, groups of 3 different injection doses were compared to aCSF vehicle group. In a control experiment to test for cortagine effects on baseline defensive behavior in the absence of the rat, a high dose cortagine group was compared to a vehicle control group. To test for receptor specificity of cortagine effects, cortagine was applied after prior blockade of CRF receptors with acidic astressin, and astressin pre-injection group was compared to high dose cortagine, and vehicle control groups. A single injection acidic astressin group was compared to vehicle control groups to test for effects of CRF receptor antagonism. Statistical evaluation of pharmacological effects of the cortagine injections (dose-response) were performed using one-way ANOVA with post-hoc Newman-Keuls test applied (Prizm 5, Graphpad, La Jolla, CA). Baseline effects of cortagine vs. vehicle were evaluated with Student's t-test. Interactions between Cortagine and acidic astressin (receptor specificity) were analyzed with two-way ANOVA (factors: agonist vs antagonist) and post-hoc Newman Keuls test (Statistica 9, StatSoft, Tulsa, OK). Non-parametric data were analyzed with Mann-Whitney U tests, or Kruskal-Wallis tests, and subsequent post-hoc Dunn's Multiple Comparison tests.

Results

Line crossings

One-way ANOVA indicated a significant effect of cortagine on line crossings (transitions from one compartment of the RET box to another) [$F(3,30)=23.87$, $p<0.0001$]. Post hoc Newman-Keuls test revealed that cortagine dose-dependently suppressed line crossings at doses of 10 ng ($p<0.001$), 50 ng ($p<0.0001$), and 120 ng ($p<0.0001$) when compared with aCSF-injected controls (see Figure 3). A high dose of cortagine (120 ng) significantly reduced line crossings also in the absence of the rat ($p<0.05$, Student's t-test). Two-way ANOVA indicated significant interaction of acidic astressin and cortagine [$F(1,31)=24.74$, $p<0.0001$], with Newman-Keuls test revealing that pre-injection of acidic astressin reliably prevented cortagine effects on line crossings ($p<0.001$). Acidic Astressin alone did not significantly alter line crossings.

Avoidance

Cortagine significantly affected location preference as measured by time spent in each compartment of the RET (Chamber, $[F(3,30)=9.21, p<0.001]$; tunnel, $[F(3,30)=16.96, p<0.0001]$; surface, $[F(3,30)=7.33, p=0.001]$), and number of entries into the compartment (Chamber, $[F(3,30)=28.33, p<0.0001]$; tunnel, $[F(3,30)=23.89, p<0.0001]$; surface, $[F(3,30)=18.1, p<0.0001]$). While the two lower doses (10 ng, 50 ng) of cortagine had no effect, the high dose of cortagine (120 ng) significantly reduced the time mice spent on the surface ($p<0.0001$), as well as in the tunnel ($p<0.0001$), while enhancing the time spent in the chamber ($p<0.0001$, see Figure 4). Cortagine dose-dependently decreased the number of entries onto the surface and into the tunnel (10 ng, $p<0.05$; 50 ng, $p<0.001$; 120 ng, $p<0.0001$), while the frequency of chamber entries (10 ng, $p<0.001$; 50 ng, $p<0.001$; 120 ng, $p<0.0001$) was increased (see Table 1). With no perceived predator threat present (no rat condition), cortagine-injected animals, compared with vehicle controls, did not spend significantly more time in the escape chamber compartment, or on the surface. Mann-Whitney U test revealed reduced tunnel time ($p<0.05$) in the cortagine group (see Table 2). Cortagine also reduced the number of entries into each compartment (Chamber, $p<0.05$; tunnel, $p<0.05$; surface, $p<0.05$; Mann-Whitney U tests). Two-way ANOVA indicated significant interaction effects of cortagine and acidic astressin on duration (DUR) and frequency (FRE) of chamber [DUR, $F(1,31)=11.56, p<0.001$; FRE, $F(1,31)=22, p<0.001$], tunnel [DUR, $F(1,31)=13.71, p<0.001$; FRE, $F(1,31)=24.61, p<0.0001$], and surface [DUR, $F(1,31)=13.33, p<0.001$; FRE, $F(1,31)=23.1, p<0.0001$] measures. While injection of acidic astressin alone, when compared to aCSF controls, did not produce significant effects on location preference, pre-injection of acidic astressin blocked cortagine effects on chamber (DUR, $p<0.001$; FRE, $p<0.001$), tunnel (DUR, $p<0.001$; FRE, $p<0.001$), and surface (DUR, $p<0.05$; FRE, $p<0.001$), measures.

Kruskal-Wallis tests indicated that additional avoidance measures such as contact with [DUR, $H(3,30)=16.57, p<0.001$; FRE, $H(3,30)=21.47, p<0.0001$], and climbing on [DUR, $H(3,30)=9.21, p<0.05$; FRE, $H(3,30)=10.51, p<0.05$] the divider mesh were also significantly affected by cortagine treatment. Post-hoc Dunn's test revealed significant reduction of contact (see Figure 5) and climbing duration ($p<0.0001$, and $p<0.05$, respectively), as well as frequency ($p<0.0001$, and $p<0.05$, respectively) in the high dose group. Similarly, in the absence of the rat, cortagine reduced contact with ($p<0.05$, Mann-Whitney U test), and climbing on the mesh ($p<0.05$, Mann-Whitney U test). Pre-injection of acidic astressin prevented cortagine effects on contact (DUR, $p<0.05$; FRE, $p<0.05$). No significant effect of acidic astressin pre-injection was detectable on climbing duration or frequency. Kruskal-Wallis ANOVA indicated that duration of burying/tunnel blocking behavior was significantly altered by cortagine [$H(3,30)=10.23, p<0.05$], and post hoc Dunn's test revealed a significant difference between high dose cortagine and aCSF groups ($p<0.05$). One-way ANOVA pointed to a significant effect of cortagine on frequency of burying/tunnel blocking behavior [$F(3,30)=3.31, p<0.05$], with Newman-Keuls test again showing a significant reduction of burying in the high dose cortagine group only ($p<0.05$). Whereas acidic astressin alone had no effect when compared with aCSF controls, pre-injection of acidic astressin blocked the effect of cortagine on duration ($p<0.001$, Dunn's test) as well as frequency ($p<0.001$, Dunn's test) of burying/tunnel blocking behavior.

Freezing

A Kruskal-Wallis test indicated a significant effect of cortagine on duration of freezing behavior [$H(3,30)=16.94, p<0.001$], and post-hoc Dunn's revealed reliable increase in duration ($p<0.001$) of freezing. Frequency of freezing behavior was also significantly affected by cortagine treatment [$F(3,34)=77.15, p<0.0001$], with Newman-Keuls showing a strong increase in frequency of freezing in the high dose group ($p<0.001$, see Figure 6). When no rat was presented, cortagine and vehicle groups did not differ significantly in their freezing behavior. Acidic astressin did not alter freezing when injected alone, but pre-injection of acidic

astressin prevented the increase in freezing duration ($p < 0.0001$, Dunn's test) and frequency induced by cortagine ($p < 0.0001$, Newman-Keuls test).

Risk assessment

Whereas statistical analysis using one-way ANOVA did not indicate any effects of cortagine on risk assessment behavior as indicated by duration of stretch-attend behavior (SAP), a slightly significant effect was indicated on frequency of SAP [$F(3,34) = 4.03$, $p < 0.05$]. However, post-hoc Newman-Keuls Multiple Comparison test revealed no significant differences between cortagine-injected mice and aCSF controls. Interestingly, in the absence of the rat, cortagine-injected mice exhibited significantly increased duration ($p < 0.001$, Mann-Whitney U test) and frequency of SAP ($p < 0.05$, Mann-Whitney U test), when compared to vehicle-treated controls.

Discussion

The present study explored the role of central CRF receptor subtype 1 (CRF1) in a mouse model of defensive behavior under semi-natural conditions, the RET. In a pharmacological approach to investigate CRF1-mediated effects on defensive behaviors, we used the recently developed, peptidic CRF1 receptor agonist cortagine, which exhibits improved selectivity for CRF1 over previously used agonists such as ovine CRF (oCRF). To test for the specificity of cortagine effects, we blocked cortagine action on CRF1 by pre-injection of acidic astressin, a peptidic CRF receptor antagonist.

Overall, injection of cortagine into the cerebroventricular system resulted in pronounced increase in avoidance behavior, combined with greatly enhanced freezing. Cortagine-injected mice showed a marked preference for staying in the chamber compartment, thereby keeping the greatest possible distance between themselves and the predatory threat presented by the rat. This avoidance behavior mediated by cortagine occurred specifically in response to the perceived threat of the rat, because when no rat was present, cortagine-injected mice clearly did not avoid the surface area. However, other measures that indicated lack of avoidance such as contact with, and climbing on the mesh separating mouse and rat compartments were virtually non-existent after cortagine injection with or without the rat present. Risk assessment as indicated by stretch-attend postures (SAP) directed toward the rat was relatively low, and slightly reduced by a medium dose of cortagine. Interestingly, when no rat was present, cortagine markedly increased SAP. This enhanced risk assessment behavior could have been stimulated by the open space of the surface area in which, in the absence of the rat, cortagine-injected mice spent much more time than when a rat was present. An alternative explanation would be that risk assessment was suppressed by increasing threat level, i.e. the presence of the rat mediating higher anxiety levels. This specific interaction of threat level and CRF1 activation by cortagine is in accordance with recent hypotheses of CRF1 function (Zhao et al., 2007), which state that with increasing anxiety levels, CRF1 mediates 'non-coping'. In the RET, this 'non-coping', which we would interpret as enhanced passive coping is expressed by reduced SAP as well as impaired burying. In the high dose, cortagine reduced burying behavior in order to block the tunnel that connected the 'escape' chamber with the 'danger' surface-zone close to the rat. The CRF receptor antagonist acidic astressin reliably blocked cortagine-induced behavioral changes. Cortagine's selectivity of CRF1 was previously investigated with i.c.v. and site specific injections of CRF2 antagonists (Tezval et al, 2004). Results from these experiments show that cortagine effects were not blocked by the CRF2-specific antagonist anti-sauvagine-30. Thus, in the present study we attribute the observed inhibition of cortagine's behavioral effects by acidic astressin to antagonism of CRF1. However, the lack of effects of acidic astressin when injected alone suggests that endogenous activation of CRF receptors does not play a major role in mediating defensive behavior in the RET, a finding that is in line with previous studies using non-selective CRF receptor antagonists in different tests of experimental

anxiety (Baldwin et al., 1991; Britton et al., 1986). We cannot rule out that alternatively, unspecific action of acidic astressin on CRF1 and CRF2 could have masked potential anxiolytic-like effects solely mediated by CRF1 blockade.

Our results are in good agreement with previous studies, which show direct enhancement of anxiety-like and defensive behavior by pharmacological CRF1 activation, and reduced anxiety-like behavior in mice deficient of CRF1. Those earlier studies (for review see Eckart et al., 1999; Radulovic et al., 1999) employed widely used laboratory tests of experimental anxiety such as the EPM and the open field, tests that measure a relatively narrow range of defensive behaviors specific to the experimental setup, such as avoidance of open arms in the EPM, and thigmotaxis in the open field. The RET advantageously stimulates a variety of innate defensive behaviors, including active/passive avoidance, freezing, and risk assessment. These behaviors belong to the innate repertoire of defensive murine behavior, but are only exhibited under unrestricted conditions and in response to a 'naturalistic' threat, e.g. a predator. In contrast, other standard laboratory tests of experimental fear and anxiety typically limit the diversity of the animal's behavior in response to the threatening stimulus, due to relatively small contexts providing no place to escape or hide. In addition, 'artificial' threats or stressors such as footshock typically evoke a very limited range of behavioral responses in rodents (for review see Litvin et al., 2008). This might also explain the somewhat unexpected unchanged fear conditioning (i.e. normal freezing responses to a conditioned tone) in CRF1-deficient mice reported in an earlier study from our laboratory (Tovote et al., 2005). Consequently, we are currently investigating the effects of CRF1-deficiency on defensive behavior in the RET.

The behaviors observed in the RET can be analyzed individually, but taken together also constitute certain patterns, indicating a specific 'type' or profile of defensive behavior. The pattern induced by i.c.v. cortagine in the presence of the perceived predatorial threat was clearly characterized by enhanced passive avoidance. Overall, cortagine changed coping with the presented threat from active strategies to pronounced passivity. Activity levels were greatly reduced, and passive avoidance, as well as freezing markedly enhanced. The shift to passive coping strategies was further indicated by the finding that more active measures of defense such as risk assessment (indicated by stretch-attend) and active avoidance (indicated by burying/tunnel blocking) were, in the presence of the rat, slightly reduced by cortagine injection. As stated before, this shift seems to be dependent on the perceived threat level, and especially in the case of risk assessment in the RET. This could have contributed to earlier findings of cortagine-induced increase in risk assessment (Farrokhi et al., 2007). These effects could be attributable to the five-fold differences of dosage (120 ng vs. 500 ng), or alternatively to the use of a different mouse strain (outbred Swiss-Webster instead of C57BL/6J), which would have had a lower threat perception. Congruent with the latter assessment, an early study using the RET demonstrated that the Swiss-Webster strain tends to exhibit more SAP, when compared to C57BL/6J (Yang et al., 2004). Similar to our high dose of 100 ng, the high dose of 500 ng cortagine used by Farrokhi et al. produced an increase in freezing and passive avoidance. Our results using i.c.v. injections of cortagine are comparable to results obtained in the RET with injections of cortagine (Litvin et al.), or oCRF (Carvalho-Netto et al.) into the periaqueductal gray (PAG). Intra-PAG activation of CRF1 by cortagine or oCRF resulted in enhanced defensiveness in the RET, as indicated by enhanced avoidance. Interestingly, neither oCRF nor cortagine affected freezing when injected into the PAG. While oCRF injected directly into the PAG reduced risk assessment, cortagine markedly enhanced burying behavior. In our experiments, i.c.v. cortagine, in the presence of the rat, had only mild effects on risk assessment and burying/tunnel blocking behavior, while risk assessment was enhanced in the no rat condition. These differences suggest a site-specific, and emotional state-dependent action of CRF1 mediated defensive behaviors: Under high anxiety-like states, CRF1-activation in brain regions likely reached by i.c.v. injections (i.e. forebrain circuitries), results in avoidance and freezing, thereby enhancing passive defensive behavior, while active forms of

defense such as risk assessment are mediated through CRF1 under lower anxiety-like states. Burying/tunnel blocking in the RET is mediated by CRF1 in the PAG. This interpretation is supported by a previous study showing increased conditioned passive avoidance after i.c.v. administration of CRF (Fekete et al., 1985). Further support comes from the study of Zhao et al. (2007) that used i.c.v. application of stressin₁-A, a different peptidic CRF1 agonist in the Shock-Probe Test in rats. Although stressin₁-A at lower doses resulted in somewhat more active defensive behavior, higher doses mediated increased freezing, similar to our findings. In addition, the CRF system has recently been reported to mediate passive coping behavior with separation stress in voles, using i.c.v.-injections of CRF receptor antagonists (Bosch et al., 2008).

In summary, our results demonstrated the usefulness of the rat exposure test in studying the effects of anxiogenic drugs on detailed patterns of defensive behaviors under semi-natural conditions. Instead of analyzing a singular behavioral indicator of fear or anxiety as commonly done in other tests, comprehensive patterns of defensive behavior can be detected with the RET. Our study confirms the strong pharmacological action of the recently developed CRF1 agonist cortagine on anxiety-like behavior *in vivo*, and supports a role for central CRF1 in mediating defensive behavior. In addition, our data suggest that cortagine, under high anxiety-like states, impairs active coping (such as risk assessment and active avoidance) with a stressful naturalistic threat and enhances passive coping strategies such as freezing, and passive avoidance.

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Figure 1.

Setup of the RET box. Chamber is connected to the surface by a tunnel section made of clear Plexiglas. Mouse surface compartment is separated from rat compartment by stainless steel mesh. Some of the mouse's home cage bedding material (sawdust) was spread out on the surface and 60 ml were provided inside the chamber for burying or blocking of the tunnel entrance.

Figure 2. Schedule of experiments. Mice were habituated to the RET box on two subsequent days before the test day to reduce novelty-induced behavior.

Figure 3.

Sums of line crossings from one compartment of the RET box (chamber, tunnel, or surface) into another in the RET. C57BL/6J mice were injected with different doses of CRF1-selective agonist cortagine, CRF receptor antagonist acidic astressin, or both, and compared with aCSF-injected controls. Effects of cortagine were also tested in the absence of a rat. Cortagine dose-dependently decreased activity, an effect that was prevented by pre-injection of acidic astressin. $n=8-10$, except $n=5$ for 50 ng cortagine group. ** $p<0.001$, *** $p<0.0001$ vs. aCSF controls; ### $p<0.001$ vs. cortagine 120 ng; § $p<0.05$ vs. aCSF controls (no rat condition).

Figure 4.

Total time spent in the escape chamber compartment (upper panel) and number of entries into the chamber (lower panel) as measures of avoidance behavior indicated by location preference. C57BL/6J mice were injected with different doses of CRF1-selective agonist cortagine, CRF receptor antagonist acidic astressin, or both, and compared with aCSF-injected controls. Effects of cortagine were also tested in the absence of a rat. While cortagine dose-dependently increased avoidance, astressin alone did not affect avoidance and blocked cortagine-induced enhancement of avoidance. With no rat present, cortagine did not enhance avoidance behavior. $n=8-10$, except $n=5$ for 50 ng cortagine group. ** $p<0.001$, *** $p<0.0001$ vs. aCSF controls; ### $p<0.0001$, ## $p<0.001$ vs. cortagine 120 ng. $^{\$}$ $p<0.05$ vs. aCSF controls (no rat condition).

Figure 5.

Total duration of contact with the divider mesh (upper panel) and number of individual contacts (frequency; lower panel) exhibited by C57BL/6J mice injected with different doses of CRF1-selective agonist cortagine, CRF receptor antagonist acidic astressin, or both, compared with aCSF controls. Cortagine significantly suppressed duration and frequency of contact in the presence or absence of the rat. $n=8-10$, except $n=5$ for 50 ng cortagine group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. aCSF controls; # $p<0.05$ vs. cortagine 120 ng. §§ $p<0.001$ vs. aCSF controls (no rat condition).

Figure 6.

Total duration of freezing (upper panel) and number of individual freezing episodes (frequency; lower panel) of C57BL/6J mice injected with different doses of CRF1-selective agonist cortagine, CRF receptor antagonist acidic astressin, or both, compared with aCSF-injected controls. Cortagine massively enhanced freezing, an effect that was reversed by pre-injection of acidic astressin. With no rat present, cortagine did not significantly increase freezing behavior. $n=8-10$, except $n=5$ for 50 ng cortagine group. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$ vs. aCSF controls; ### $p<0.0001$, ## $p<0.001$ vs. cortagine 120 ng.

Duration and frequency of RET-behaviors of mice injected i.c.v. with different doses of CRF1-selective agonist cortagine (corta), compared with vehicle (aCSF) controls to determine dose-response effects.

Table 1

Duration	aCSF	corta 10 ng	corta 50 ng	corta 120 ng	ac. ast.	ac. ast. + cortagina
Chamber	281.5 ± 38.1	305.3 ± 34.2	310.3 ± 82.3	543.7 ± 22.3 ***	344.2 ± 36.2	313.3 ± 33.2##
Tunnel	64.3 ± 4.8	80.7 ± 7.5	57.2 ± 8.8	17.8 ± 6.5 ***	81.3 ± 8.8	91.9 ± 10.0##
Surface	253.4 ± 38.7	212.3 ± 33.5	232.3 ± 78.1	27.0 ± 7.8 **	173.0 ± 36.5	193.3 ± 35.9#
Freezing	11.7 ± 2.2	7.7 ± 1.8	15.8 ± 8.0	88.7 ± 16.7 **	30.2 ± 10.9	13.1 ± 3.3###
SAP	24.0 ± 5.0	15.5 ± 5.3	15.5 ± 6.8	26.6 ± 4.6	21.8 ± 4.1	13.9 ± 3.2
Burying/Tunnel blocking	18.8 ± 4.5	35.3 ± 13.8	20.8 ± 7.4	2.1 ± 1.5 *	27.7 ± 10.3	32.8 ± 9.0##
Contact	79.2 ± 16.3	46.2 ± 15.7	71.6 ± 28.1	1.1 ± 0.5 ***	39.4 ± 18.8	33.4 ± 11.9#
Climbing	19.1 ± 8.4	20.6 ± 13.6	47.6 ± 25.3	0.0 ± 0.0 *	18.1 ± 11.4	11.9 ± 6.5

Frequency	aCSF	corta 10 ng	corta 50 ng	corta 120 ng	ac. ast.	ac. ast. + cortagina
Chamber	20.9 ± 1.9	13.8 ± 1.1 **	9.2 ± 1.6 ***	3.3 ± 0.6 ***	21.8 ± 2.2	29.6 ± 4.4##
Tunnel	39.2 ± 4.0	25.0 ± 2.7	17.6 ± 4.2	3.1 ± 0.5 ***	38.5 ± 4.7	52.7 ± 7.6##
Surface	19.5 ± 2.3	11.9 ± 1.6	9.2 ± 2.5	1.0 ± 0.0 ***	17.4 ± 2.7	24.3 ± 3.7##
Freezing	7.9 ± 1.4	2.9 ± 0.8	4.2 ± 2.1	34.1 ± 2.3 ***	13.4 ± 2.4	9.0 ± 1.4###
SAP	13.0 ± 2.6	5.8 ± 1.5	4.4 ± 1.0 *	13.0 ± 2.1	16.6 ± 2.8	11.3 ± 2.3
Burying/Tunnel blocking	7.1 ± 1.3	4.8 ± 1.8	3.6 ± 1.4	1.3 ± 0.8 *	7.9 ± 2.5	14.8 ± 3.3#
Contact	29.5 ± 5.6	9.1 ± 1.7 **	8.4 ± 2.3 **	1.3 ± 0.6 ***	11.9 ± 4.0	13.8 ± 3.1
Climbing	2.1 ± 0.6	0.9 ± 0.5	2.4 ± 1.3	0.0 ± 0.0 *	1.8 ± 1.0	2.0 ± 1.0

* p<0.05,

** p<0.001,

*** p<0.0001.

p<0.05,

Receptor specificity of effects are depicted by comparison of single- and pre-injection groups of antagonist acidic astressin with high dose cortagine and vehicle control.

p<0.001,
p<0.0001.

Table 2

Duration and frequency of RET-behaviors of mice injected i.c.v. with CRF1-selective agonist cortagine (corta), compared with vehicle (aCSF) controls in absence of a rat to determine baseline effects of the pharmacological treatment.

	Duration		Frequency	
	aCSF	corta 120 ng	aCSF	corta 120 ng
Chamber	121.0 ± 26.2	167.8 ± 91.0	16.4 ± 3.5	4.7 ± 1.9 ^{\$}
Tunnel	66.7 ± 13.5	22.7 ± 8.8 ^{\$}	24.9 ± 4.5	5.286 ± 2.101 ^{\$\$}
Surface	383.1 ± 31.3	408.6 ± 94.6	13.6 ± 2.0	4.0 ± 1.7 ^{\$\$}
Freezing	7.0 ± 4.6	17.09 ± 3.2	8.0 ± 1.2	3.4 ± 1.8
SAP	3.5 ± 1.7	89.6 ± 23.6 ^{\$\$}	2.6 ± 1.0	25.3 ± 4.6 ^{\$\$}
Burying/Tunnel blocking	44.1 ± 9.3	19.5 ± 10.2	7.4 ± 1.1	4.3 ± 1.9
Contact	42.1 ± 7.5	0.6 ± 0.6 ^{\$\$}	5.7 ± 0.8	0.3 ± 0.3 ^{\$\$}
Climbing	17.1 ± 5.3	0.5 ± 0.5 ^{\$\$}	6.3 ± 1.4	0.3 ± 0.3 ^{\$\$}

^{\$} p<0.05,

^{\$\$} p<0.001.