Effects of acidic-astressin and ovine-CRF microinfusions into the ventral hippocampus on defensive behaviors in rats

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Abstract

This study investigated a possible role for ventral hippocampal corticotropin-releasing factor (CRF) in modulating both unconditioned and conditioned defensive behaviors by examining the effects of pre-training ventral hippocampal ovine-CRF (oCRF) or acidic-astressin ($[Glu^{11,16}]$Ast) microinfusions in male Long Evans hooded rats exposed to various threat stimuli including the elevated plus-maze (EPM) (oCRF), cat odor (oCRF and $[Glu^{11,16}]$Ast) and a live cat ($[Glu^{11,16}]$Ast). Unconditioned defensive behaviors were assessed during threat exposure, while conditioned defensive behaviors were assessed in each predator context 24 h after the initial threat encounter. Pre-training infusions of the CRF_1 and CRF_2 receptor agonist oCRF significantly increased defensive behaviors during both the unconditioned and conditioned components of the cat odor test, as well as exposure to the EPM. In contrast to the behavioral effects of oCRF microinfusions, the CRF_1 and CRF_2 receptor antagonist $[Glu^{11,16}]$Ast significantly decreased defensive behaviors during exposure to cat odor, while producing no discernible effects following a second injection in the cat exposure test. During conditioned test trials, pre-training infusions of $[Glu^{11,16}]$Ast also significantly reduced defensive behaviors during re-exposure to both predator contexts. These results suggest a specific role for ventral hippocampal CRF receptors in modulating anxiety-like behaviors in several ethologically relevant animal models of defense.

Keywords

CRF; Hippocampus; Defense; Anxiety; Fear; Conditioning; Predator; EPM; Rats; Stress

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Introduction

Small mammalian prey species such as rats and mice have developed specific species-typical antipredator defensive behaviors to facilitate predator detection (e.g., risk assessment) and increase avoidance (e.g., flight, freezing) of threat sources (Apfelbach et al., 2005; Blanchard and Blanchard, 2003, 1989; Blanchard and Blanchard, 1989). In laboratory settings, evaluation of antipredator defensive behaviors allows for an ethological analysis of fear and anxiety by exposing animals directly to a natural predator or potential threat sources, respectively. The defensive behaviors associated with these ethologically relevant threat stimuli can be divided into two broad categories based upon the intensity and/or ambiguity of the threat stimulus. In the presence of an immediate threat source, such as a live cat, flight (if possible) or freezing is the primary response pattern, while confrontation with ambiguous/potential threats, such as cat odor or predator-paired contexts, elicits risk assessment (Blanchard et al., 1989; Blanchard and Blanchard, 1989, 1988). Those defensive behaviors evoked during predator exposure have been used as an index of fear, while risk assessment behaviors, responsive to standard anxiolytics such as diazepam and $5-HT_{1A}$ agonists, have been used as an index of anxiety (Blanchard et al., 2003, 2001; Griebel, et al., 1999a, 1999b).

Recent studies aimed at examining the neurocircuitry controlling the expression of unconditioned defensive behaviors have begun to focus on a possible modulatory role for the ventral hippocampus (VH) (Bertoglio et al., 2006; Pentkowski et al., 2006). Pentkowski and colleagues (2006) demonstrated that excitotoxic VH lesions failed to reliably alter behavioral responses to a live cat, but reduced defensiveness to its odor, suggesting a preferential role in mediating certain anxiety-like behaviors. In support of this assertion, VH lesions using either lidocaine (Bertoglio et al., 2006), tetrodotoxin (Degroot and Treit, 2004), ibotenic acid (Kjelstrup et al., 2002) or electrolytic techniques (Trivedi and Coover, 2004; Bannerman et al., 2002), all produced anxiolytic-like profiles in the elevated plus-maze (EPM). Concurrently, numerous VH lesion studies have demonstrated anxiolytic-like effects in the shock-probe burying, social interaction, light/dark and hyponeophagia tests of defense (Bannerman et al., 2003; Degroot & Treit, 2004; McHugh et al., 2004), with these results resembling the anxiolytic-like effects induced by benzodiazepines in many of the same behavioral tests (Menard & Treit, 1999; Gray & McNaughton, 2000). Collectively these results indicate that the neural circuitry mediating the expression of unconditioned defensive behaviors includes the VH.

In addition to modulating defensive responses to unconditioned threat stimuli, the VH also contributes to the expression of conditioned fear. Pre-training VH ibotenic acid lesions reduced the expression of contextual fear 24 h after exposure to cat odor, a live cat or footshock (Pentkowski et al., 2006). Similarly, reversible VH lesions using tetrodotoxin, the GABA receptor agonist muscimol (Bast et al., 2001) or the NMDA receptor antagonist MK-801 (Zhang et al., 2001), all reduced the expression of conditioned fear, while permanent pre-training excitotoxic VH lesions produced deficits in cue elicited conditioned freezing (Richmond et al., 1999). Collectively, these findings suggest that in addition to a role in modulating unconditioned defense behaviors, the VH may subserve the expression of conditioned fear.

The neuropeptide corticotropin-releasing factor (CRF) represents a possible mediator of the behavioral responses modulated by the VH during the aforementioned tests of defense. CRF is a 41 amino-acid peptide that functions to mediate the autonomic, behavioral, endocrine and immune responses to stress, via actions at two G protein-coupled receptor subtypes termed CRF_1 and CRF_2 (Hillhouse and Grammatopoulos, 2006; Carrasco and Van de Kar, 2003; Spiess et al.,1981). In rats, central CRF_1 receptor mRNA expression is found throughout the isocortex, cerebellum, periaqueductal gray, amygdala and hippocampus, whereas CRF_2

receptors are located predominantly in the lateral septum, ventromedial hypothalamus, bed nucleus of the stria terminalis, dorsal raphe nucleus and olfactory bulb, with moderate levels located throughout the hippocampus (Van Pett et al., 2000; Chalmers et al., 1995).

Overall, evidence has been relatively consistent for a role of CRF_1 receptors in modulating anxiety-like defensive behaviors. Intracerebroventricular (i.c.v.) administration of the CRF agonists human/rat CRF (h/rCRF) or ovine-CRF (oCRF) increased fear or anxiety-like defensive behaviors during the acoustic startle (Risbrough et al., 2004), defensive withdrawal (Heinrichs and Joppa, 2001) and social interaction (Campbell et al., 2004) tests. Antalarmin, a non-peptidic CRF_1 receptor antagonist, blocked the anxiogenic-like effects of CRF in the EPM, decreased defensive withdrawals in a brightly lit open field, and blocked the motor-activating effects of CRF without causing sedation (Zorrilla et al., 2002), indicating heightened defensiveness in response to central CRF_1 receptor activation (Bale and Vale, 2004). However, while the central effects of CRF_1 receptors appear consistent, the behavioral effects of site-specific microinjections of various CRF compounds have produced conflicting results (Todorovic et al., 2005; Bale and Vale, 2004). Injections of urocortin I, a CRF_1 and CRF_2 receptor agonist, into the basolateral amygdala (BLA) produced anxiogenic-like effects in the social interaction test (Spiga et al., 2006), while dorsal hippocampal and lateral septum infusions of h/rCRF were found to enhance and impair conditioned freezing (Radulovic et al., 1999), respectively. Collectively these results suggest that global activation or inactivation of CRF_1 receptors via i.c.v. administration increases and decreases defensive behavior, respectively, while indicating regional specificity for the behavioral role of CRF_1 receptors (Henry et al., 2006; Radulovic et al., 1999).

In contrast to the consistent anxiogenic-like effects produced by central CRF_1 receptor activation, the precise role of CRF_2 receptors remains less clear (Todorovic et al., 2005; Bale and Vale, 2004). Activation of CRF_2 receptors via i.c.v. injections of urocortin II, a preferential CRF_2 receptor agonist, produced delayed anxiolytic-like effects in the EPM (Valdez et al., 2002), open field and light/dark (Venihaki et al., 2004) tests. Conversely, central urocortin II administration enhanced anxiogenic-like responses in the EPM, marble burying and open field tests of defense (Pelleymounter et al., 2002, 2004). The behavioral effects of centrally administered CRF_2 receptor antagonists also appear to be inconsistent, as central infusions of the preferential CRF_2 receptor antagonist antisauvagine (aSVG-30) or inhibition of CRF_2 receptor expression with antisense oligonucleotides both reduced conditioned freezing and produced anxiolytic-like effects in the EPM and defensive withdrawal tests (Ho et al., 2001). However, i.c.v. infusions of aSVG-30 have also been shown to produce an anxiogenic profile similar to CRF_2 receptor knockouts (Todorovic et al., 2005; Kishimoto et al., 2000). These behavioral differences following various CRF manipulations may involve CRF acting on different neural circuits that mediate different defensive behaviors (Lowry and Moore, 2006), and indicate the importance of determining the effects of site-specific CRF manipulations on unconditioned as well as conditioned defensive behaviors related to fear and anxiety (Takahashi, 2001; Todorovic et al., 2005).

The present studies sought to examine the role of ventral hippocampal CRF receptors in modulating defensive behaviors, hypothesizing that microinfusions of the CRF agonist oCRF would increase defensiveness during exposure to the EPM and cat odor, while administration of the CRF antagonist acidic-astressin ([Glu[11,16]]Ast) would decrease similar responses to cat odor and a live cat. The EPM was substituted for cat exposure in the defensive test battery evaluating oCRF infusions, as cat exposure consistently produces an asymptotic freezing response, which would not have allowed for a measurable increase in defensive behavior, as expected with oCRF (Blanchard et al., 2005). In addition, considering the extensive body of evidence suggesting that the VH plays a crucial role in modulating both unconditioned and conditioned defensive behaviors, contextual fear conditioning for each of the predator tests

was also examined by exposing the same subject to the relevant test chamber 24 h after the initial threat encounter. oCRF and [Glu[11,16]]Ast were used for these experiments based on similar selectivity for CRF receptors, improved solubility and higher bioavailability levels compared to other CRF compounds such as h/rCRF and astressin (Eckart et al., 2001). For example, h/rCRF and astressin possess over 100 fold higher affinity for the CRF binding protein compared to oCRF (Sutton et al., 1995) and [Glu[11,16]]Ast (Eckart et al., 2001), respectfully, allowing us to use lower doses/volumes to target CRF receptors. Furthermore, the active dose levels of h/rCRF and astressin at CRF receptors would likely vary since it would depend on the amount bound by the binding protein.

Materials and Methods

The housing conditions and care of the animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All testing procedures conducted on subjects in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Hawaii at Manoa. All efforts were made to minimize any animal pain and/or suffering.

Subjects

Subjects were male Long-Evans hooded rats born and reared in the Snyder Hall breeding colony at the University of Hawaii at Manoa. Animals weighed between 350 and 450 g at the time of surgery. Following weaning (21 days), all animals were singly housed under controlled temperature (21 ± 2 °C) and illumination (12 h light/dark cycle, lights on at 06:00 a.m.) with free access to food and water.

Surgery

Prior to surgery, animals were randomly assigned to either an experimental-CRF drug group (oCRF or [Glu[11,16]]Ast), or to their corresponding artificial cerebrospinal fluid (aCSF)-vehicle control group. Subjects were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg; Sigma-Aldrich) and were mounted in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was incised and retracted, and the head was positioned to place Bregma and Lambda in the same horizontal plane. Two sets of small burr holes (2.0 mm in diameter) were drilled in the skull in order to implant stainless steel guide cannulae (22G, Plastics One, Roanoke, VA) bilaterally into the VH; guide cannulae were implanted 1.0 mm dorsal to the desired injection site. Cannulae were implanted using a David Kopf micromanipulator aimed at intermediate regions of the VH using coordinates obtained from the Rat Brain Atlas (Paxinos and Watson, 1998); −5.2 mm posterior to bregma, +/− 5.1 mm from the midline and −6.0 mm ventral from the surface of the skull. Guide cannulae were affixed to the skull using dental cement and two 24 g jewelers' screws, and following implantation dummy cannulae were inserted into each guide in order to prevent blockage and/ or infection. Following surgery, animals were administered 3 ml of 0.9% saline in order to prevent dehydration and were returned to their home cage for a 1-wk recovery period prior to the start of behavioral testing.

Peptides

[Glu[11,16]]Ast (Mw 3637) and oCRF (Mw 4670) were obtained from Dr. Joachim Spiess at the Max-Planck Institute for Experimental Medicine (Gottingen, Germany) and from American Peptide Company (Sunnyvale, CA), respectively. Each peptide was diluted in an aCSF solution (CH3COOH/1xaCSF) at pH = 7.4 until the final low and high doses were obtained; 200 ng/ 0.5 μl and 400 ng/0.5 μl of [Glu[11,16]]Ast, and 100 ng/0.5 μl and 200 ng/0.5 μl of oCRF. Control animals received 0.5 μl infusions of the aCSF vehicle solution.

Peptide Infusion

Each subject was gently restrained by hand, the dummy cannulae were removed and 0.5 μl of the appropriate compound (vehicle, oCRF or [Glu[11,16]]Ast) was infused over a course of 60 s. The injectors were connected via polyethylene-20 tubing (Plastics One) to two 10 μl Hamilton microsyringes mounted in an infusion pump (Harvard Apparatus, Holliston, MA) in order to control the rate of infusion. The injectors extended 1.0 mm below the end of the guide cannulae to insure accurate peptide delivery. Before being slowly removed, injectors (Plastics One) remained in place for an additional 60 s to allow for complete drug diffusion.

Behavioral Testing

All testing was conducted between 09:00 and 12:00 h in one of the following two sequences. Subjects assigned to the oCRF experiments received vehicle or agonist infusions 15 min before testing in the EPM, and again 5 days later 15 min prior to cat odor exposure. Subjects in the [Glu[11,16]]Ast experiments were microinfused with the vehicle or antagonist 30 min prior to the cat odor test, and again 5 days later 30 min before cat exposure. Between every subject trial, each apparatus was thoroughly cleaned using a 10% ethanol solution. Unconditioned defensive behaviors were assessed during the EPM, cat odor and cat exposure tests, while conditioned defensive behaviors were assessed for both predator tests in each context 24 h after initial threat exposure. Each study (oCRF and [Glu[11,16]]Ast) was conducted using a separate group of animals, bred at different times and tested in different orders as well as partly different tests [cat odor-cat exposure [Glu[11,16]]Ast) or EPM-cat odor (oCRF)]. Therefore, variations among cat odor baseline control levels can be attributed to many varying/uncontrollable conditions.

Elevated Plus-Maze

The EPM apparatus consisted of four Plexiglas arms arranged in a cross, elevated 75 cm above the floor. Each arm was 10 cm wide and 50 cm long, and each arm was joined at the center by a 10 cm square platform. The two opposite "open" arms contained no walls, while the other two "closed" arms had 40 cm high sides. Subjects were individually placed in the center arm of the apparatus facing one of the two closed arms. All sessions were 5 min in duration and were conducted under red light.

Cat Odor Exposure

The cat odor test apparatus consisted of a white Plexiglas runway ($100 \times 12 \times 50$ cm) with a clear front panel to permit observation and recording. A cloth-wrapped solid plastic block ($9 \times 9 \times 2$ cm) was rubbed for 5 min against the fur of laboratory-housed domestic male cat for three consecutive days and was then stored in a Ziploc plastic bag until serving as the cat odor stimulus. Both control and experimental animals were habituated to the apparatus for 10 min on three consecutive days without the presence of an odor block. On the fourth day (unconditioned behavior test), the cat odor block was placed at one end of the runway and a subject was placed at the opposite end, facing away from the odor block. 24 h later subjects were retested in the same apparatus without the cat odor stimulus (conditioned behavior test). A cloth-wrapped plastic block never exposed to cat odor served as the cue during conditioned test trials. All sessions were 10 min in duration and were conducted under red light in order to ensure that the threat stimulus remained as ambiguous as possible.

Cat Exposure

The test apparatus consisted of two adjacent subject chambers ($50 \times 20 \times 30$ cm) separated by an opaque white Plexiglas wall, with a wire-mesh screen separating these chambers from the adjoining cat compartment ($55 \times 40 \times 35$ cm). Two subjects were simultaneously placed, one in each subject chamber, facing away from the cat compartment (the same cat used in the cat

odor test). Following a 5 min pre-cat exposure period, the cat was introduced into the cat compartment for 10 min (unconditioned behavior test). 24 h hours later, subjects were retested in the same apparatus without the cat stimulus (conditioned behavior test). Each test was conducted under white light to ensure the cat stimulus was as unambiguous as possible.

Behavioral Measures

Dependent measures analyzed in each test situation included *freezing*-complete cessation of movement other than respiration; *grooming*-movement of forepaws or tongue over the body; *transits*-crossing of lines marking the far, medium and near thirds of the apparatus (cat odor and cat exposure), or the number of open and closed arm entries (EPM), measured as any movement from one marked section of the apparatus to another; *avoidance*-duration of time spent in the far compartment relative to the threat stimulus (cat odor: block; cat exposure: wire-mesh), or the proportion of time (duration) spent in the closed versus open arms (EPM). Additional behavioral measures scored in both the cat odor and cat exposure tests included *rearing*-standing on rear paws with forepaws raised off the ground; and *crouchsniff*- olfactory investigation evidenced by vertical or lateral head movements, scoring initiated when nose visibly moved more than 1 cm. *Head-dips*-extension of the subjects head over the edge of an open arm; and *risk assessment*-combined measure of both *stretch approach*-forward ambulation with flat back and stretched neck, and *stretch attend*-standing on all four paws with flat back and stretched neck orientated toward the threat source-were additional measures scored in the EPM. Behavioral measures represent the durations of events in seconds (or numbers of transits) in either a 5 (EPM) or 10 min (cat odor and cat exposure) observation period.

Behavioral Analysis

All test trials were recorded using Pioneer DVD recorders, and were later analyzed using the behavioral analysis software Observer 5.0 (Noldus Information Technology, Wageningen, The Netherlands) by a highly trained observer blind to drug conditions. Use of this software allowed for a detailed, frame-by-frame analysis.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed on each dependent measure in the EPM, cat odor and cat exposure paradigms; α was set at 0.05. Post-hoc Newman-Keuls tests were conducted in order to compare drug and vehicle control means; α was set at 0.05. In several instances where ANOVA approached but failed to reach statistical significance, planned comparisons using Dunnett's test were conducted to test for significant differences between drug and control means.

Histology

Following the completion of behavioral testing, histological verification of cannulae placement was performed. All subjects were overdosed with sodium pentobarbital (100 mg/kg), and infused with 0.5 μl of 1% methylene blue using identical procedures as those during initial drug administration. Subjects were then perfused transcardially with 0.9% saline followed by 4% formalin. Following extraction from the skull, brains were placed into a 4% formalin solution for 48 h and then transferred to a 30% sucrose solution until blocking (at least 48 h) and sectioning on a cryostat (Leica). A series of coronal sections throughout the entire rostral–caudal extent of each cannulae placement were taken (40 μm thick, collected every 80 μm). After drying (24 h), the sections were analyzed under a microscope (Leica, USA) in order to verify cannulae tip placement.

Results

Histology

Figure 1 presents representative photomicrographs of injector tip placements within the VH for rats included in both the oCRF and [Glu[11,16]]Ast experiments. Only those subjects with correct cannulae placements (injector tip) located in the VH within the anterior–posterior range defined as −4.8 to −5.6 mm posterior to bregma, the medial–lateral range defined as ±4.8 to 6.0 mm lateral to the midsagittal suture, and the dorsal–ventral range defined as −5.0 to −7.0 mm ventral to the flat skull surface were included in the analyses (Paxinos and Watson, 1998). Three rats from the oCRF vehicle (n=9), two from the 100 ng (n=9) and three from the 200 ng (n=9) oCRF groups were excluded due to placements outside the circumscribed region. One additional subject from each oCRF drug group was excluded from the cat odor analysis due to a lost head cap (n=8 for each oCRF drug group; n=9 for vehicle controls). Three rats from the [Glu[11,16]]Ast vehicle (n=8), three from the 200 ng (n=9), and two from the 400 ng (n=8) drug groups were excluded due to misplaced cannulae tips outside the VH; one additional 200 ng subject (n=8) was excluded from the cat exposure analysis due to a lost head cap. Table 1 presents data from animals with cannulae tip placements located outside of the aforementioned range. These data illustrate ventral hippocampal specificity for the effects of oCRF and [Glu[11,16]]Ast injections, as opposed to that at neighboring regions; subjects did not differ from vehicle controls (F>0.05 in each case).

Elevated Plus-Maze (oCRF)

The data for both the behavioral and proximal measures in the EPM following pre-exposure VH oCRF microinfusions are summarized in figure 2. One-way ANOVA indicated that oCRF reliably decreased the percentage of time spent in the open arms [$F_{(2, 24)}=8.2380$, $p<0.005$], with Newman-Keuls post-hoc tests revealing that both the 100 ng and 200 ng groups spent less time in the open arms compared to vehicle controls ($p<0.005$ in each case); there were no significant differences between the two oCRF drug groups. oCRF infusions significantly decreased head dips [$F_{(2,24)}=6.3904$, $p<0.01$], with Newman-Keuls showing that both the 100 ng and 200 ng doses reduced levels compared to vehicle controls ($p<0.01$); there was no reliable difference between 100 ng and 200 ng oCRF groups. One-way ANOVA revealed a significant oCRF dose effect on risk assessment [$F_{(2,24)}=7.1937$, $p<0.005$], with subsequent Newman-Keuls indicating that the 200 ng dose increased durations compared to both 100 ng ($p<0.01$) and vehicle control ($p<0.005$) groups; there were no reliable differences between the 100 ng and vehicle groups. VH oCRF infusions failed to significantly alter the number of transits (data not shown).

Cat Odor Exposure (oCRF)

The data for both the behavioral and proximal measures in the cat odor exposure test following pre-exposure VH oCRF microinfusions are summarized in figure 3. One-way ANOVA revealed a significant main effect of drug on the duration of freezing [$F_{(2, 22)}=6.9520$, $p<0.005$], with subsequent post-hoc Newman-Keuls tests showing that oCRF infusions produced a dose-dependent increase in freezing as 200 ng dosed rats exhibited higher levels than both the 100 ng ($p<0.05$) and vehicle control ($p<0.005$) groups; there was no reliable difference between the 100 ng and vehicle groups. Analysis of crouch-sniff duration using a one-way ANOVA indicated a near significant effect of drug [$F_{(2, 22)}=3.1712$, $p=.06$]. Planned comparisons indicated that subjects receiving the 200 ng, but not 100 ng, dose of oCRF displayed significantly lower durations of crouch-sniff compared to vehicle controls ($p<0.05$). The durations of grooming (data not shown), rearing and avoidance were not reliably different between groups, and there was no significant difference in the number of transits (data not shown).

Cat Odor Exposure Cue + Context Conditioning (oCRF)

The data for both the behavioral and proximal measures in the cat odor exposure cue + context conditioning test following pre-training VH oCRF infusions are summarized in figure 3. One-way ANOVA revealed a significant drug effect on the duration of freezing [$F_{(2,22)}=6.3801$, $p<0.01$], with Newman-Keuls post-hoc analysis indicating increased levels for the 200 ng group compared to vehicle controls ($p<0.01$); subsequent pair-wise comparisons failed to show further group differences. Analysis of rearing using a one-way ANOVA demonstrated a near significant main effect [$F_{(2, 22)}=2.8692$, $p=0.07$]. Planned comparisons indicated a reliable reduction in the duration of rearing following infusions of 200 ng ($p<0.05$), but not 100 ng, of oCRF. VH oCRF infusions failed to reliably alter the durations of avoidance, crouch-sniff, grooming (data now shown) or the number of transits (data now shown).

Cat Odor Exposure ([Glu^{11,16}]Ast)

The data for both the behavioral and proximal measures in the cat odor exposure test following pre-exposure VH [Glu^{11,16}]Ast microinfusions are summarized in figure 4. One-way ANOVA revealed a significant main effect of [Glu^{11,16}]Ast on the duration of freezing [$F_{(2, 22)}=12.326$, $p<0.0005$], with subsequent Newman-Keuls post-hoc tests showing that both the 200 ng ($p<0.005$) and 400 ng ($p<0.0005$) groups froze less than vehicle controls; there were no reliable differences between drug groups. One-way ANOVA revealed a significant dose effect on the duration of rearing [$F_{(2,22)}=5.9372$, $p<0.01$], with Newman-Keuls showing that 200 ng infusions increased rearing durations compared to vehicle controls ($p<0.05$), while 400 ng approached, but failed to reach significance ($p=.05$); there were no reliable differences between [Glu^{11,16}]Ast drug groups. There was a significant main effect of [Glu^{11,16}]Ast on the number of transits [$F_{(2, 22)}=5.4878$, $p<0.05$] (data now shown), with Newman-Keuls showing increased levels for both the 200 ng ($p<0.05$) and 400 ng ($p<0.01$) drug groups; there were no reliable differences between [Glu^{11,16}]Ast dose groups. One-way ANOVA indicated that [Glu^{11,16}]Ast infusions did not produce reliable group differences in the durations of crouch-sniff or grooming (data now shown). Analysis of [Glu^{11,16}]Ast infusions using a one-way ANOVA revealed a significant main effect on the duration of avoidance [$F_{(2, 22)}=4.1469$, $p<0.05$], with post-hoc Newman-Keuls revealing that 400 ng infusions significantly reduced avoidance compared to vehicle controls ($p<0.05$); subsequent pair-wise comparisons failed to show further group differences.

Cat Odor Exposure Cue + Context Conditioning ([Glu^{11,16}]Ast)

The data for both the behavioral and proximal measures in the cat odor exposure cue + context conditioning test following pre-training VH [Glu^{11,16}]Ast microinfusions are summarized in figure 4. One-way ANOVA demonstrated that [Glu^{11,16}]Ast infusions significantly reduced the duration of conditioned freezing [$F_{(2, 22)}=3.8997$, $p<0.05$], with post-hoc Newman-Keuls revealing a dose dependent effect as 400 ng dosed animals froze less than vehicle controls ($p<0.05$); 100 ng infusions approached but failed to reach significance ($p=.06$) and there were no differences between the two drug groups. [Glu^{11,16}]Ast significantly increased the number of transits [$F_{(2, 22)}=4.0429$, $p<0.05$] (data now shown), with Newman-Keuls showing that rats receiving the 400 ng dose exhibited more line crossings than both the vehicle control ($p<0.05$) and 200 ng groups, although the latter effect was not reliable ($p=0.05$); there were no differences between the 200 ng and vehicle control groups. One-way ANOVA indicated that there were no reliable differences in the durations of crouch-sniff, rearing or grooming (data now shown). One-way ANOVA indicated that differences in the duration of avoidance approached, but did not reach, statistical significance ($p=.06$).

Cat Exposure ([Glu^{11,16}]Ast)

The data for both the behavioral and proximal measures in the cat exposure test following pre-exposure VH [Glu^{11,16}]Ast microinfusions are summarized in figure 5. [Glu^{11,16}]Ast failed to produce significant differences between vehicle and drug dose groups on any of the behavioral measures during the cat exposure test.

Cat Exposure Context Conditioning ([Glu^{11,16}]Ast)

The data for both the behavioral and proximal measures in the cat exposure context conditioning test following pre-training VH [Glu^{11,16}]Ast microinfusions are summarized in figure 5. One-way ANOVA revealed that[Glu^{11,16}]Ast significantly reduced conditioned freezing [$F(2, 21)=5.6600$, $p<0.05$], with Newman-Keuls post-hoc analysis indicating that both drug groups exhibited lower levels relative to vehicle controls ($p<0.05$ in each case); there were no significant differences between the two drug groups. There was a significant main effect of [Glu^{11,16}]Ast on the duration of crouch-sniff [$F(2, 21)=4.8712$, $p<0.05$], with 400 ng of [Glu^{11,16}]Ast increasing durations compared to vehicle controls ($p<0.05$); subsequent pair-wise comparisons failed to show further group differences. There were no reliable effects of [Glu^{11,16}]Ast infusions on the durations of grooming (data now shown), rearing, avoidance or the number of transits (data now shown).

Discussion

The present results indicate that ventral hippocampal CRF receptors modulate both unconditioned and conditioned behaviors in several ethologically relevant tests of defense. Activation of ventral hippocampal CRF receptors following site-specific microinfusions of oCRF increased defensive behaviors during exposure to potential (EPM, cat odor and cat odor context) threats, while inhibition of CRF receptors following [Glu^{11,16}]Ast microinfusions decreased defensive behaviors to potential (cat odor and both predator contexts), but not present (cat exposure), threat stimuli. While other studies have evaluated the effects of intra-hippocampal CRF infusions (Blank et al., 2002; Radulovic et al., 1999; Lee and Davis, 1997) this is the first *in-vivo* study to specifically evaluate the function of endogenous ventral hippocampal CRF receptors in mediating the expression of unconditioned defensive behaviors, as well as the role of these receptors during conditioning to the aforementioned predatory threats.

Ventral hippocampal oCRF infusions produced robust increases in anxiety-like defensive behaviors in the EPM, reducing the proportion of time spent in the open-arms and the number of head dips, while dose dependently increasing the duration of risk assessment, without altering locomotion. Following a second injection, oCRF also potentiated defensive behaviors during exposure to cat odor, dose dependently increasing and decreasing the durations of freezing and sniffing, respectively, suggesting that oCRF shifted the pattern of defensiveness such that the behavioral responses elicited by cat odor resembled those appropriate if heightened levels of environmental threat were present (i.e. a live predator). In contrast to the anxiogenic-like profile produced by oCRF, microinfusions of [Glu^{11,16}]Ast preferentially attenuated defensive behaviors during exposure to cat odor, reducing the duration of freezing to, and avoidance of, the cat odor stimulus, while increasing the number of approaches to the odor block (transits); a shift in defensive responsivity similar to reducing the level of environmental threat (i.e. a return to non-defensive behaviors). During live cat exposure, [Glu^{11,16}]Ast failed to alter any behavioral response, indicating that antagonism of VH CRF receptors reduces defensiveness during exposure to potential threats, but not to a present predator. The lack of an effect following VH [Glu^{11,16}]Ast infusions during cat exposure is consistent with lesion data (Pentkowski et al., 2006) suggesting that the modulation of defensive behaviors during exposure to a potent immediate threat is not VH dependent, and

that other neural systems can support defensiveness to a clearly present predator, in the absence of VH activity. Alternatively, during the cat-exposure test [Glu[11,16]]Ast injected animals may have been less defensive to olfactory cues, while the additional auditory and visual cues of the live cat stimulus were sufficiently potent as to elicit a near maximal freezing response.

Pre-training antagonism of ventral hippocampal CRF receptors also affected the expression of conditioned defensive behaviors. Similar to the effects of VH lesions (Pentkowski et al., 2006; Trivedi and Coover, 2004), pre-training microinfusions of [Glu[11,16]]Ast reduced conditioned defensive behaviors during re-exposure to both predator-paired contexts, with oCRF administration producing the opposite effect during the cat odor cue + context conditioning test. Pre-training [Glu[11,16]]Ast infusions dose-dependently decreased the duration of freezing and increased the number of approaches to the non-odor block cue (transits), while reducing and increasing the durations of freezing and sniffing, respectively, during re-exposure to the cat context. In cat odor cue + context conditioning test trials, pre-training oCRF infusions potentiated defensive behaviors in a dose dependent manner, increasing and decreasing the durations of freezing and rearing, respectively. This enhancement or attenuation in the expression of defensive behavior following pre-training oCRF or [Glu[11,16]]Ast microinfusions, respectively, suggests that the formation of conditioned fear involves VH CRF receptor activation. These changes in the expression of conditioned defensive behaviors may have resulted from changes in levels of defensiveness during the unconditioned test trials, or changes in situational learning from altered VH functioning (Blank et al., 2002).

In each unconditioned and conditioned test situation oCRF and [Glu[11,16]]Ast failed to alter non-defensive behaviors (grooming), suggesting a preferential role for ventral hippocampal CRF receptors in mediating the modulatory role of the VH during exposure to potential threats, including cat odor, contexts associated with predators (Pentkowski et al., 2006) and the EPM (Bertoglio et al., 2006). Although the increase in transits (locomotion) during both the unconditioned and conditioned cat odor tests following ventral hippocampal [Glu[11,16]]Ast microinjections could indicate general locomotor effects of the drug, results from the cat exposure test argue against this possibility. During unconditioned test trials, both [Glu[11,16]]Ast and vehicle infused animals froze for almost the entire duration of the test (figure 4), and increases in general locomotion would be anticipated following a reduction in levels of freezing. Furthermore, numerous studies have demonstrated anxiolytic-like effects without accompanying changes in locomotion following inactivation of the VH (Pentkowski et al., 2006; Degroot and Treit, 2004; Bannerman et al., 2003; Bast and Feldon, 2003; Kjelstrup et al., 2002).

The contrasting effects following [Glu[11,16]]Ast infusions during cat odor and cat exposure may indicate that VH CRF receptors mediate defensive behaviors sensitive to tests of anxiety, without affecting behaviors responsive to tests of fear. In support of this notion, Blanchard & Blanchard (1990) suggested that situations in which rats are exposed to specific immediate threat stimuli (cat exposure) elicit fear-like responses, whereas tests exposing rats to situations of potential or anticipated threat (cat odor, cat-paired contexts) elicit anxiety-like behaviors, an assertion supported by pharmacological data (Blanchard et al., 2003). For instance, classic anxiolytics such as the benzodiazepines diazepam and chlordiazepoxide (Blanchard et al., 1990c), alcohol (Blanchard et al., 1990a), the 5-HT$_{1A}$ agonists 8-OH-DPAT and Gepirone (Blanchard et al., 1992) and the tricyclic antidepressant imipramine (Blanchard et al., 1993) decrease freezing and avoidance while increasing risk assessment during exposure to contexts associated with a cat, while the benzodiazepine midazolam decreases freezing and avoidance during cat odor exposure (McGregor et al., 2004). In contrast, during exposure to an unavoidable predator diazepam failed to reliably alter levels of risk assessment or freezing, but

again increased measures of risk assessment immediately following predator removal (Blanchard et al., 1990c).

The biological mechanisms of endogenous hippocampal CRF may explain the behavioral effects produced by CRF manipulations in the current studies. Recent research has implicated CRF in hippocampal cellular excitation (Blank et al., 2002; Kortekaas et al., 1999), as *in-vitro* application of h/rCRF to hippocampal slice preparations primed long-term potentiation (LTP) of population spikes (Blank et al., 2002), which was prevented by pre-application of [Glu^{11,16}]Ast. Furthermore, Blank and colleagues (2002) showed that this priming was required for contextual fear conditioning, which may explain the current effects of CRF during the conditioning test trials. *In-vivo* CRF infusions in anesthetized rats have also been shown to increase hippocampal theta activity (Kortekaas et al., 1999), a mechanism believed to be involved in emotional regulation (McNaughton et al., 2007; Gray and McNaughton, 2000), particularly in modulating anxiety-like defensive behaviors (Deroot and Treit, 2002). In fact, all known classes of drugs that are clinically effective in treating generalized anxiety disorder (e.g. barbiturates, benzodiazepines, 5-HT$_{1A}$ receptor agonists, SSRIs) decrease the frequency of reticular-elicited theta, while drugs that are antipsychotic or sedative but not anxiolytic (haloperidol, chlorpromazine) do not have this effect (McNaughton et al, 2007). Collectively, these electrophysiological results suggest that the current effects obtained following CRF infusions may have resulted from VH neural excitation (oCRF) or inhibition ([Glu^{11,16}]Ast), with the latter finding corroborated from numerous studies showing reduced defensiveness following inactivation of the VH (Bertoglio et al., 2006; Pentkowski et al., 2006; Degroot and Treit, 2004; McHugh et al., 2004; Bannerman et al., 2002).

Neuroanatomically the VH could modulate unconditioned and conditioned defensive behaviors via direct or indirect efferent projections to various hypothalamic and/or amygdaloidal nuclei. Intermediate regions of ventral CA1 and the subiculum project to the BLA, lateral, medial (MeA) and posterior basomedial amygdalar nuclei (Canteras & Swanson, 1992; Petrovich et al., 1996, 2001; Pikkarainen et al., 1999), structures all implicated in mediating various aspects of unconditioned and conditioned defense (Phelps and LeDoux, 2005; Rosen, 2004). Of these, the MeA is of particular importance in relation to cat odor, as it receives heavy projections from the vomeronasal organ, a structure known to play a critical role in the detection of predator odors, and lesions restricted to the MeA dramatically reduce defense behaviors during cat odor exposure (Blanchard et al., 2005; Li et al., 2004). Intermediate regions of ventral CA1 and the subiculum also send efferent projections indirectly to the hypothalamus via projections through the dorsal region of the ventrolateral zone of the rostral part of the lateral septal nucleus (Risold and Swanson, 1997) to the anterior hypothalamic nucleus and dorsal premammillary nucleus [PMd] (Canteras et al., 2001; Comoli et al., 2000; Risold and Swanson, 1997). These hypothalamic structures, along with the dorsomedial portion of the ventromedial hypothalamus, form the medial hypothalamic zone, a core circuit hypothesized to play a central role in the integration and initiation of defensive behaviors to innate threat stimuli (Canteras, 2002). Indeed recent research has supported a role for this circuit in modulating unconditioned defensive behaviors to natural predatory threats with PMd lesions abolishing freezing to both a live cat and its odor (Markham et al., 2004; Blanchard et al., 2003; Canteras et al., 1997).

Using the fear potentiated startle paradigm, Lee and Davis (1997) failed to demonstrate increased conditioned fear following oCRF microinfusions into the VH, a finding seeming at variance with the present results. However, they used lower doses (40 ng and 80 ng) and volumes (0.3 μl) than the present study, and a somewhat different infusion location: The current study targeted intermediate regions of ventral CA1 (main source of amygdaloidal and hypothalamic projections), while the Lee and Davis (1997) study targeted anterior ventral regions of the VH. Additionally, the current study infused oCRF rather than h/rCRH,

suggesting that the heightened cat odor induced conditioned defensiveness may have been due to increased CRF_1 receptor activation as oCRF is more selective for CRF_1 than CRF_2 receptors and possesses lower affinity for the CRF binding protein in comparison to h/rCRF (Radulovic et al., 2001). Finally, inconsistencies obtained between these studies could be due to differences in testing conditions, specifically the use of footshock pain as the unconditioned threat stimulus in the Lee and Davis (1997) study.

Conclusion

The effects of ventral hippocampal oCRF and [Glu[11,16]]Ast microinjections on defensive behaviors in Long-Evans hooded rats were evaluated in the present studies. Our findings demonstrate that oCRF potentiated, while [Glu[11,16]]Ast attenuated, defensive behaviors in a situation-specific, dose-related manner, suggesting that activation of endogenous ventral hippocampal CRF receptors modulate the expression of unconditioned defensive behaviors during testing in paradigms more closely related to anxiety (anticipated or potential threat) rather than fear (immediate and clear threat). Furthermore, these results indicate that endogenous ventral hippocampal CRF receptors mediate conditioning processes related to predatory threats.

Abbreviations

aCSF	artificial cerebrospinal fluid
aSVG-30	antisauvagine
BLA	basolateral amygdala
CRF	corticotropin-releasing factor
EPM	elevated plus-maze
[Glu[11,16]]Ast	acidic-astressin
h/rCRF	human/rat CRF
i.c.v	intracerebroventricular
LTP	long-term potentiation
oCRF	ovine-CRF
PMd	dorsal premammillary nucleus
VH	ventral hippocampus

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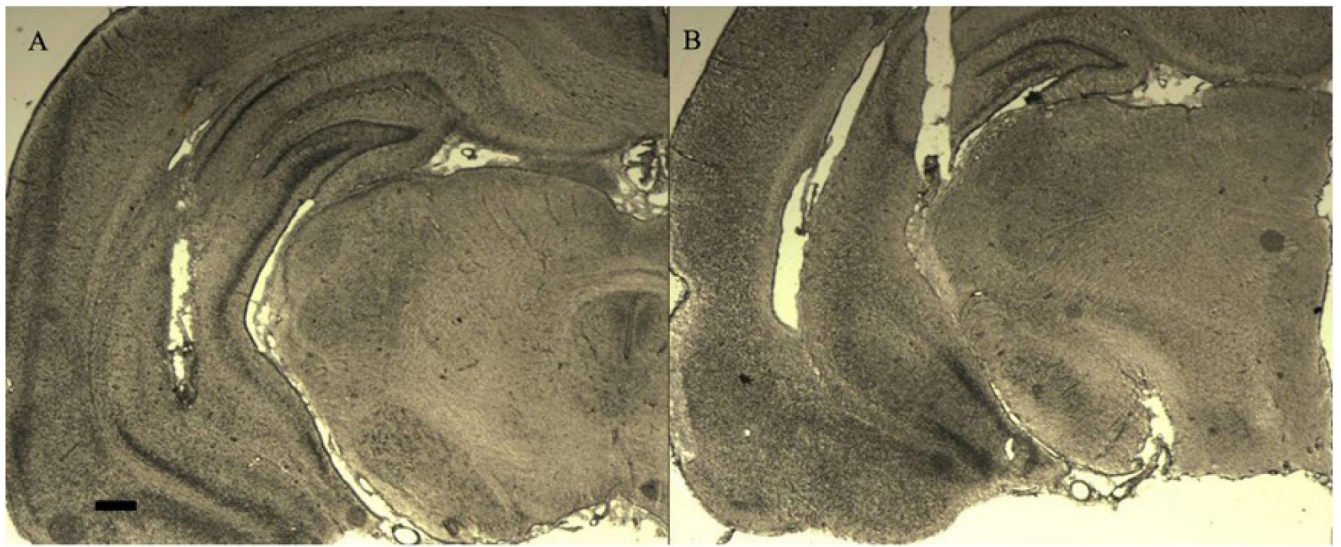
Figure 1.
Photomicrographs of representative ventral hippocampal cannulae placements included (A) and excluded (B) in the analysis. Scale bar, 420 μm.
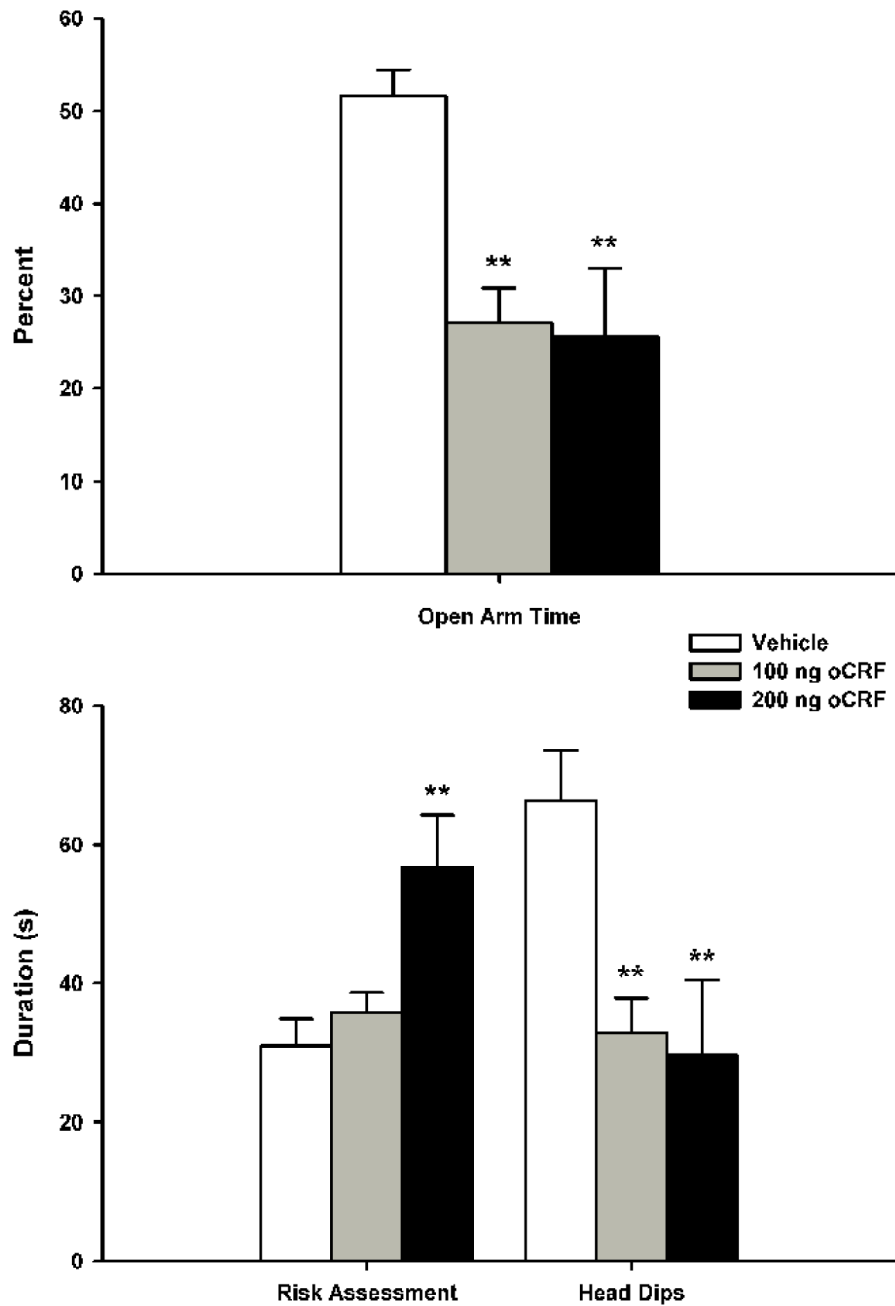
Figure 2.
Effects of ventral hippocampal oCRF infusions (mean + S.E.M.) on defensive behaviors during the EPM test trials. Animals (n=9/group) received their assigned dose of either vehicle or oCRF 15 min prior to testing. Differences for which p<0.01[**] compared to vehicle controls (Newman-Keuls).
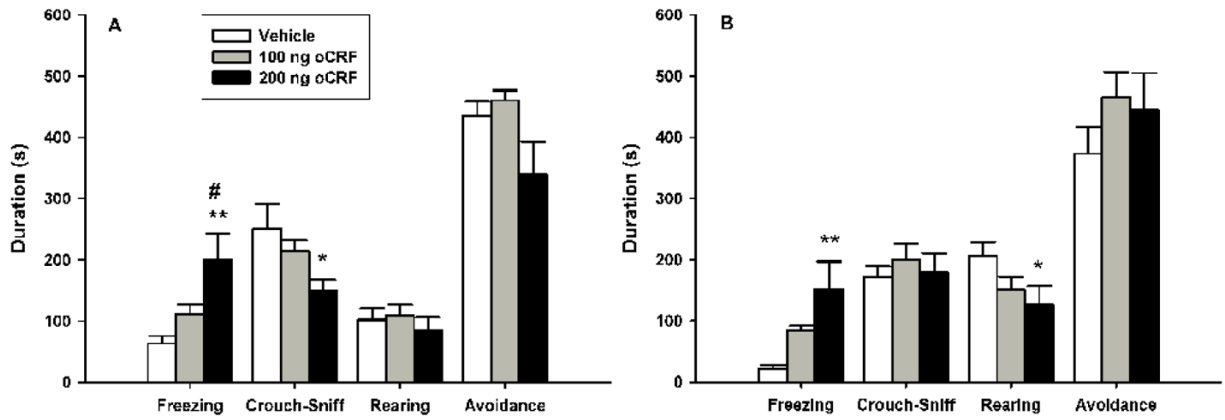
Figure 3.
Effects of ventral hippocampal oCRF infusions (mean + S.E.M.) on defensive behaviors during the cat odor (A) and cat odor cue + context conditioning (B) test trials. Animals (n=8/group) received their assigned dose of either vehicle or oCRF 15 min prior to testing. Differences for which $p<0.05$[*] and $p<0.01$[**] compared to vehicle controls; $p<0.05$[#] compared to the 100 ng drug group (Newman-Keuls).
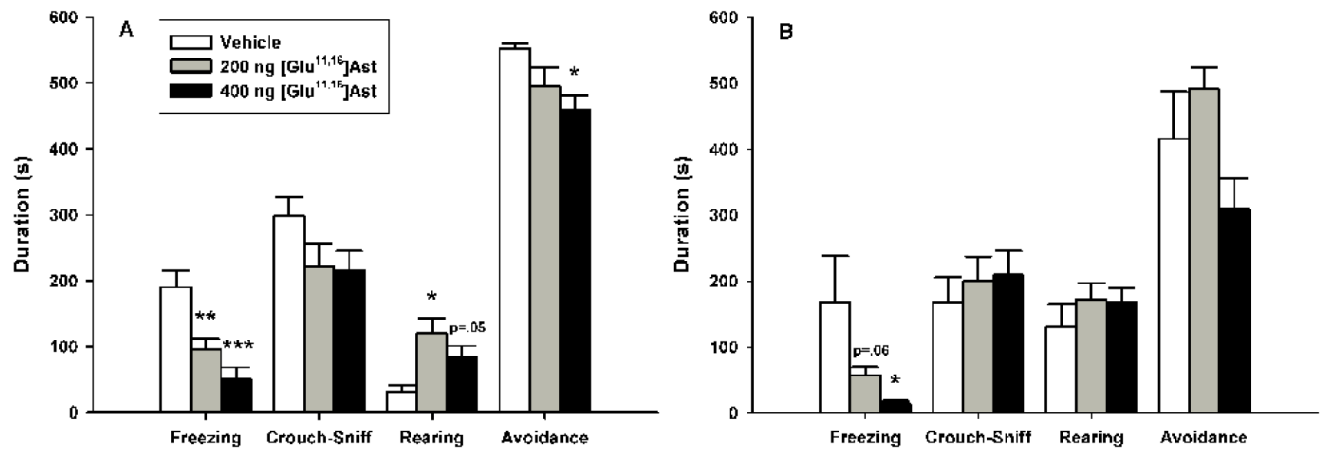
Figure 4.
Effects of ventral hippocampal [Glu^{11,16}]Ast infusions (mean+S.E.M.) on defensive behaviors during the cat odor (A) and cat odor cue + context conditioning (B) test trials. Animals (n=8–9/group) received their assigned dose of either vehicle or [Glu^{11,16}]Ast 30 min prior to testing. Differences for which p<0.05*, p<0.01** and p<0.001*** compared to vehicle controls (Newman-Keuls).
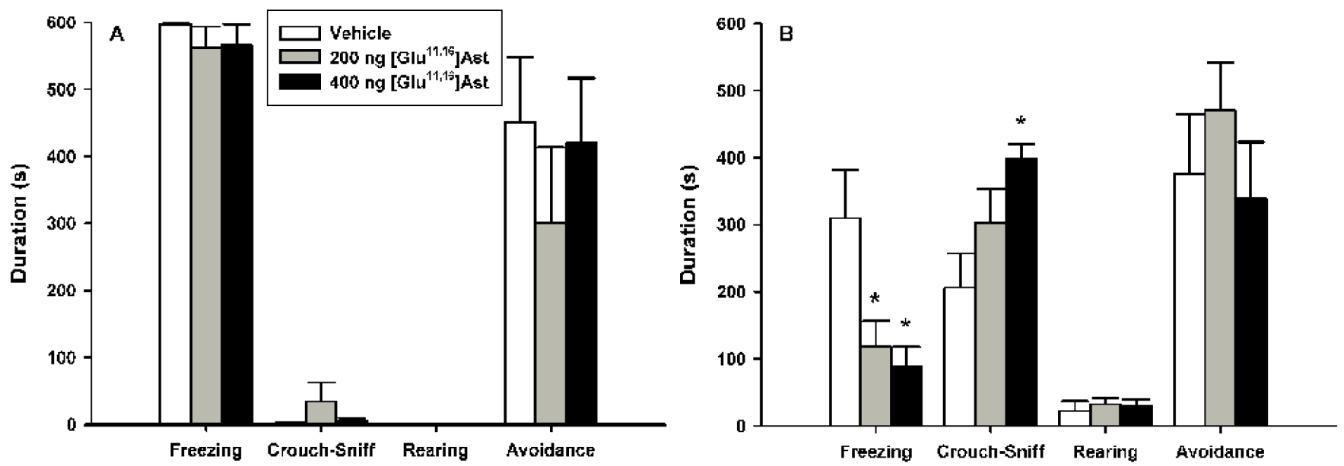
Figure 5.
Effects of ventral hippocampal [Glu^{11,16}]Ast infusions (mean + S.E.M.) on defensive behaviors during the cat exposure (A) and cat exposure context conditioning (B) test trials. Animals (n=8/group) received their assigned dose of either vehicle or [Glu^{11,16}]Ast 30 min prior to testing. Differences for which p<0.05* compared to vehicle controls (Newman-Keuls).

Table 1

Selected behaviors in the EPM, cat odor, cat odor cue + context conditioning, cat exposure and cat exposure context conditioning tests from subjects that received oCRF and [Glu^{11,16}]Ast injections, yet whose cannulae were in regions outside of the VH. There were no reliable differences between CRF drug and vehicle control groups.

Elevated Plus Maze	
Behavioral Measures	oCRF
Percent Open Arm Time	0.62 ± 0.13
Risk Assessment (s)	26.00 ± 7.69
Head Dips (s)	62.00 ± 28.78

	Cat Odor Exposure	Cue + Context Conditioning
Behavioral Measures	oCRF	oCRF
Freezing (s)	64.80 ± 28.65	23.90 ± 5.67
Crouch-Sniff (s)	188.80 ± 35.30	176.60 ± 29.64
Rearing (s)	137.90 ± 25.84	210.20 ± 16.72
Avoidance (s)	401.60 ± 54.46	417.40 ± 55.30

	Cat Odor Exposure	Cue + Context Conditioning
Behavioral Measures	[Glu^{11,16}]Ast	[Glu^{11,16}]Ast
Freezing (s)	232.00 ± 47.75	136.20 ± 64.82
Crouch-Sniff (s)	226.40 ± 36.91	192.60 ± 35.89
Rearing (s)	43.20 ± 18.97	117.60 ± 48.65
Avoidance (s)	540.90 ± 16.89	381.50 ± 105.84

	Cat Exposure	Context Conditioning
Behavioral Measures	[Glu^{11,16}]Ast	[Glu^{11,16}]Ast
Freezing (s)	599.25 ± 0.75	419.00 ± 78.05
Crouch-Sniff (s)	0.00 ± 0.00	160.63 ± 67.35
Rearing (s)	0.00 ± 0.00	4.88 ± 3.57
Avoidance (s)	450.25 ± 150.08	580.75 ± 12.41