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Stress and central Urocortin increase anxiety-like behavior in the social interaction test via the CRF_1 receptor

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Abstract

Corticotropin releasing factor (CRF) and Urocortin are important neurotransmitters in the regulation of physiological and behavioral responses to stress. Centrally administered CRF or Urocortin produces anxiety-like responses in numerous animal models of anxiety disorders. Previous studies in our lab have shown that Urocortin infused into the basolateral nucleus of the amygdala produces anxiety-like responses in the social interaction test. Subsequently, in the current study we prepared a specific CRF_1 receptor antagonist (*N*-Cyclopropylmethyl-2,5-dimethyl-*N*-propyl-*N*'-(2,4,6-trichloro-phenyl)-pyrimidine-4,6-diamine, NBI3b1996) to examine in this paradigm. This CRF_1 receptor antagonist inhibited the ex vivo binding of ^{125}I-sauvagine to rat cerebellum with an ED_{50} of 6 mg/kg, i.p. NBI3b1996 produced a dose-dependent antagonism of Urocortin-induced anxiety-like behavior in Social Interaction test with an ED_{50} of 6 mg/kg, i.p. The compound had no effect on baseline social interaction. In addition, the CRF_1 receptor antagonist prevented the stress-induced decrease in social interaction. These results provide further support for the CRF_1 receptor in anxiety-like behavior and suggest this pathway is quiescent in unstressed animals.

Keywords: Corticotropin releasing factor; Stress; Behavior; Amygdala; Antagonist; (Rat)

1. Introduction

The 41 amino acid peptide, corticotropin releasing factor (CRF) is a pituitary secretagogue for adrenocorticotropin hormone (Vale et al., 1981) and also acts as a neurotransmitter (Landgraf, 2001; Palkovits et al., 1983). Since its isolation and characterization, preclinical studies have documented the importance of CRF in the regulation of behavioral responses to stress (Bale et al., 2002; Carrasco and Van de Kar, 2003; Habib et al., 2001). Stressors produce a rapid release of CRF and intracerebroventricular administration of CRF replicates many of the behavioral and physiological effects of stress (Buwalda et al., 1997; Dirks et al., 2002; Liang et al., 1992; Matsuzaki et al., 1989). Transgenic mice that overexpress CRF exhibit heightened stress related behaviors and have elevated function of the hypothalamus–adrenal–pituitary axis while mice that have null mutations for CRF exhibited a blunted endocrine response to stress and reduced stress behaviors (Contarino and Gold, 2002). In addition to preclinical data, clinical studies have also implicated CRF in the etiology of depression and other affective disorders. Maladaptive responses to stress and chronically elevated levels of corticosteroids have been a consistent and common finding in major depression (Strohle and Holsboer, 2003). It is likely that the increased plasma levels of cortisol are attributed to increases in hypothalamic CRF and/or vasopressin. Successful depression therapy is often accompanied by reduced cerebrospinal fluid CRF concentrations and a return of plasma corticosterone levels to normal ranges (Strohle and Holsboer, 2003).

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CRF and the related peptide transmitters are believed to produce their biological effects by interaction with two G-protein coupled receptors and one binding protein (Dautzenberg and Hauger, 2002). These CRF receptor subtypes are distributed heterogeneously in the brain suggesting the potential for functional diversity. Autoradiographic localization, tissue binding and in situ hybridization studies have been utilized to show distinct regional distribution for the CRF_1 and CRF_2 receptors in the rat central nervous system (Rominger et al., 1998). In particular, the CRF_1 receptor has been proposed as an important contributor to CRF effects on anxiety-like behaviors. Mice lacking the CRF_1 receptor were reported to have reduced anxiety-like behaviors when compared to wild type controls (Timpl et al., 1998). In a carefully controlled study, Heinrichs et al. (1997) reported that antisense oligonucleotides directed at reducing central CRF_1 receptor expression reduced anxiety-like behaviors in the defensive withdrawal model. Conditional knockout of the brain, but not the pituitary, CRF_1 receptor resulted in decreased anxiety-like behaviors and a normal hypophyseal–pituitary axis (Muller et al., 2003). However, these animals exhibited an endocrine hypersensitivity to stress demonstrating the importance of brain CRF_1 receptor in mediating the behavior responses to stress and the independence of this action from the endocrine effects of CRF. In contrast to the CRF_1 receptor, the role of the CRF_2 receptor is less well understood. Nevertheless, some recent work has suggested that the CRF_2 receptor may also mediate CRF effects on anxiety-like behaviors, however, further study will be required to fully understand its importance (Takahashi, 2002).

CRF and Urocortin share similar sequence homology and are both agonists at the CRF_1 and the CRF_2 receptors (Vaughan et al., 1995). In vitro, Urocortin has a slightly greater affinity for CRF_1 and at least a fourfold greater affinity for CRF_2, while CRF has higher affinity for the CRF_1 receptor compared to the CRF_2. There has been extensive work over the last several decades focusing on the amygdala as a critical nucleus in anxiety and stress-like responses (Amaral et al., 2003; Campeau and Davis, 1995; Davis, 1998). CRF containing fibers and CRF receptors are reported to be abundant in the amygdala (Morin et al., 1999; Sawchenko et al., 1993). Recently, we reported that low doses of Urocortin and CRF infused into the basolateral nucleus of the amygdala increased anxiety-like behaviors in the rat Social Interaction test (Sajdyk et al., 1999), a fully validated animal model of anxiety (File and Seth, 2003). Pretreatment of the basolateral nucleus of the amygdala with the nonselective antagonist, astressin, antagonized the effects of Urocortin on social interaction (Sajdyk and Gehlert, 2000). In the present study, we sought to understand the role of the CRF_1 receptor in the anxiety-like responses induced by Urocortin. To accomplish this, we synthesized a potent and selective CRF_1 receptor antagonist *N*-Cyclopropylmethyl-2,5-dimethyl-*N*-propyl-*N*'-(2,4,6-trichloro-phenyl)-pyrimidine-4,6-diamine (NBI3b1996) (Example 3 b in (Chen et al., 1996); see Fig. 1). We characterized this antagonist using in vitro quantitative autoradiography and ex vivo binding to establish potency and to set doses for the behavioral studies. Then, we assessed the effects of this compound on intra-amygdalar Urocortin-induced anxiety-like behavior in the Social Interaction test as well as stress-induced reductions in social interaction.

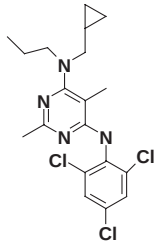

Fig. 1. Chemical structure of NBI3b1996.

2. Materials and methods

2.1. Rat forebrain autoradiography

Autoradiographic localization of ^{125}I-sauvagine binding to CRF_1 and CRF_2 receptors (Grigoriadis et al., 1996) was performed using sections of rat brain with a method modified from (Million et al., 2003). Tissue sections were preincubated at 25 °C for 30 min in modified Krebs phosphate buffer with 0.05% bacitracin (Sigma Aldrich, St. Louis, MO) and 0.4% bovine serum albumin (Sigma Aldrich, St. Louis, MO) (pH 7.4). Slides were incubated in 100 pM ^{125}I-sauvagine (Perkin Elmer/NEN, Boston, MA, 2200 Ci/mmol) for 2 h at 25 °C. Adjacent sections were incubated with the addition of various concentrations of NBI3b1996 (synthesized at Eli Lilly and Company, Indianapolis, IN). Nonspecific binding was assessed by the addition of 1 uM CRF (Bachem) to the incubation media. Sections were rinsed twice for 10 min each in buffer, then dipped twice in cold distilled water and dried quickly with a stream of cool air. Sections were exposed with ^{125}I-Microscale Standards (Amersham, Pharmacia Biotech, Piscataway, NJ) on Kodak Biomax MR-1 film (Eastman Kodak, Rochester, NY) for 3 days, then developed with Kodak D-19 developer. Quantitation was facilitated using the MCID Elite (St. Catherines, ON) image analysis. Curve fitting and ED_{50} determinations were accomplished using GraphPad Prism software (San Diego, CA).

2.2. Ex vivo binding

Male Sprague Dawley rats, 225–250 g, were injected i.p. with vehicle (1 ml/kg, 3% dimethylsulfoxide (DMSO), 20% Emulphor in water) or compound in vehicle. After 1 h, the rats were decapitated and the cerebellum was removed, frozen on dry ice and stored at

−70 °C. On the day of the assay, tissue was thawed, homogenized in buffer (50 mM Tris–HCl, 2 mM EGTA, 10 mM $MgCl_2$) and incubated at 37 °C for 1 h. For the binding assay, approximately 200 μg of prepared homogenate was combined with a final concentration of 0.175 nM ^{125}I-Tyro-Sauvagine (Perkin Elmer, Boston, MA) in assay buffer with 0.1% bovine serum albumin, 0.1% bacitracin and 100 kU/ml aprotinin. Non-specific binding was determined by the addition of 1 μM ovine CRF (American Peptide Company). After incubation at room temperature for 120 min, the assay was terminated by centrifugation and binding assessed using a gamma counter. Significance was determined by ANOVA analysis followed by Newman–Keuls post hoc test.

2.3. Surgical procedures

Experiments were conducted using Male Wistar rats (275–300 g) from Harlan Laboratories (Indianapolis, IN). The animals were individually housed and given food and water ad libitum. All animals were maintained in a temperature controlled room (21–22 °C) with a 12-h light/dark cycle schedule. Approximately 48–72 h from arrival, animals were taken to the surgical room for placement of bilateral cannulae into the basolateral nucleus of the amygdala. Animals were removed from their home cage and placed in a closed plastic holding box which was connected to an isoflurane system (MGX Research Machine, Vetamac, Inc., Rossville, IN). The animals were completely anesthetized, then placed into a stereotaxic instrument (Kopf Instruments, Tujunga, CA) with the incisor bar set at −3.3 mm. Anesthesia was maintained via a nose cone placed on the incisor bar which allowed for continuous flow of isoflurane throughout the surgery. The top of the head was shaved and cleaned with Betadine and 70% alcohol. An incision was made in the scalp and the skull was cleaned and dried. Two stainless steel guide cannulae (26 gauge, 10 mm length; Plastic Products, Roanoke, VA) were situated into guide cannulae holders fixed onto the stereotaxic arms. The guide cannulae were lowered into position of the basolateral nucleus of the amygdala using coordinates (A, −2.1; L, 5.0; V, −8.5) according the brain atlas of Paxinos and Watson (1986). The guide cannulae were secured into place using three 2.4 mm screws anchored into the skull along with cranioplastic cement. Following placement of dummy cannulae into the guide cannulae, animals were injected with Buprenex (0.25 mg/kg sc; Reckitt and Colman, Richmond, VA) and removed from the stereotaxic apparatus and allowed to recover for 72–96 h.

2.4. Behavioral assessment

Anxiety behavior was measured utilizing a modified version of the Social Interaction test where only the behavior of the experimental animal is accounted for during the 5 min test. The treated animal was simultaneously placed into the Social Interaction box (a 36″L×36″W×12″H box with an open top) with a novel partner rat. All partner rats were of the same sex and similar weight, housed under identical conditions and had no previous contact with the treated animal. Testing was carried out under low light (40 W red lighting; 0.012 klx) familiar conditions, while Social Interaction testing for anxiolytic behavior was conducted under bright light familiar conditions (34 W florescent lighting; 0.44 klx). Familiarization was accomplished by allowing the animals one 5 min session alone within the testing box. All testing was video taped via a camera mounted on the ceiling directly above the Social Interaction box and occurred between 8:00 AM and 11:30 AM. Scoring of Social Interaction times was determined as previously described (Sajdyk and Shekhar, 1997b), that is, only interactions such as sniffing grooming, play, etc. initiated by the treated rat were scored as total time interacting (s) during a 5 min trial.

2.5. Behavioral experimental procedures

2.5.1. Effect of NBI3b1996 on social interaction following Urocortin into the basolateral nucleus of the amygdala

Animals underwent surgery to implant bilateral cannulae into the basolateral nucleus of the amygdala. Seventy-two hours following surgery, on experimental day 1, the animals were administered an i.p. injection of the antagonist vehicle (1 ml/kg, 3% dimethylsulfoxide, 20% Emulphor), then 30 min later an intra-amygdalar injection of 1% bovine serum albumin (peptide vehicle). Then, 30 min later, all animals were assessed for baseline activity in the Social Interaction test. Forty-eight hours later, animals were separated into 3 groups (n=5 each). The first group received 3 mg/kg, the second group 5 mg/kg and the third group a 10 mg/kg dose of the CRF_1 receptor antagonist 30 min prior to intra-amygdalar administration of Urocortin (100 fmol). Cannulae placements were confirmed following experimentation using histological techniques. Only animals exhibiting correct cannulae placement were used for subsequent analyses. Correct cannulae placement was observed in 90% of the animals. Statistical testing was conducted only on the total time in seconds that the treated animal initiated interaction with the partner rat during the 5-min test. A paired t-test was utilized for statistical evaluation of data from the experimental groups in the dose response study. GraphPad Prism software was used to determine the ED_{50}.

2.5.2. Effect of NBI3b1996 on social interaction following restraint stress

A group of eight male Wistar rats (275–300 g) from Harlan Laboratories (Indianapolis, IN) was utilized for the restraint stress study and housed in conditions identical to those in the Social Interaction studies. One week after arrival, all animals were habituated to the Social Interaction box. Twenty-four hours later all rats were assessed for baseline social interaction times under bright light conditions. Then 24 h

later, each animal was placed in a restraint chamber (Braintree Scientific, Braintree, MA) for 30 min. This procedure was repeated daily for a total of 3 days. Forty-eight hours later the animals were divided into 4 treatment groups (vehicle, 3, 10 and 30 mg/kg NBI3b1996). On experimental day 1, each group was administered the assigned treatment and then 30 min later placed in the restraint chamber for an additional 30 min. Following restraint, all animals were assessed for behavioral effects in the Social Interaction test. Then, in a counter-balanced design, the same protocol as day 1 was repeated, with each group receiving each treatment separated by 48 h, for a total of four treatment days. Data were analyzed using a repeated measures ANOVA followed by a Newman–Keul's post hoc test. GraphPad Prism software was used to determine the ED_{50}.

All animal studies were approved by the animal care and use committees at either Eli Lilly and Company or Indiana University.

3. Results

3.1. Assessment of in vitro affinity and selectivity of NBI3b1996

Initial studies were performed to assess the potency and selectivity of NBI3b1996. To accomplish this, a series of autoradiographic experiments were performed using the nonselective radioligand ^{125}I-sauvagine (Fig. 2). In these studies, slide mounted sections of rats for brain and brainstem

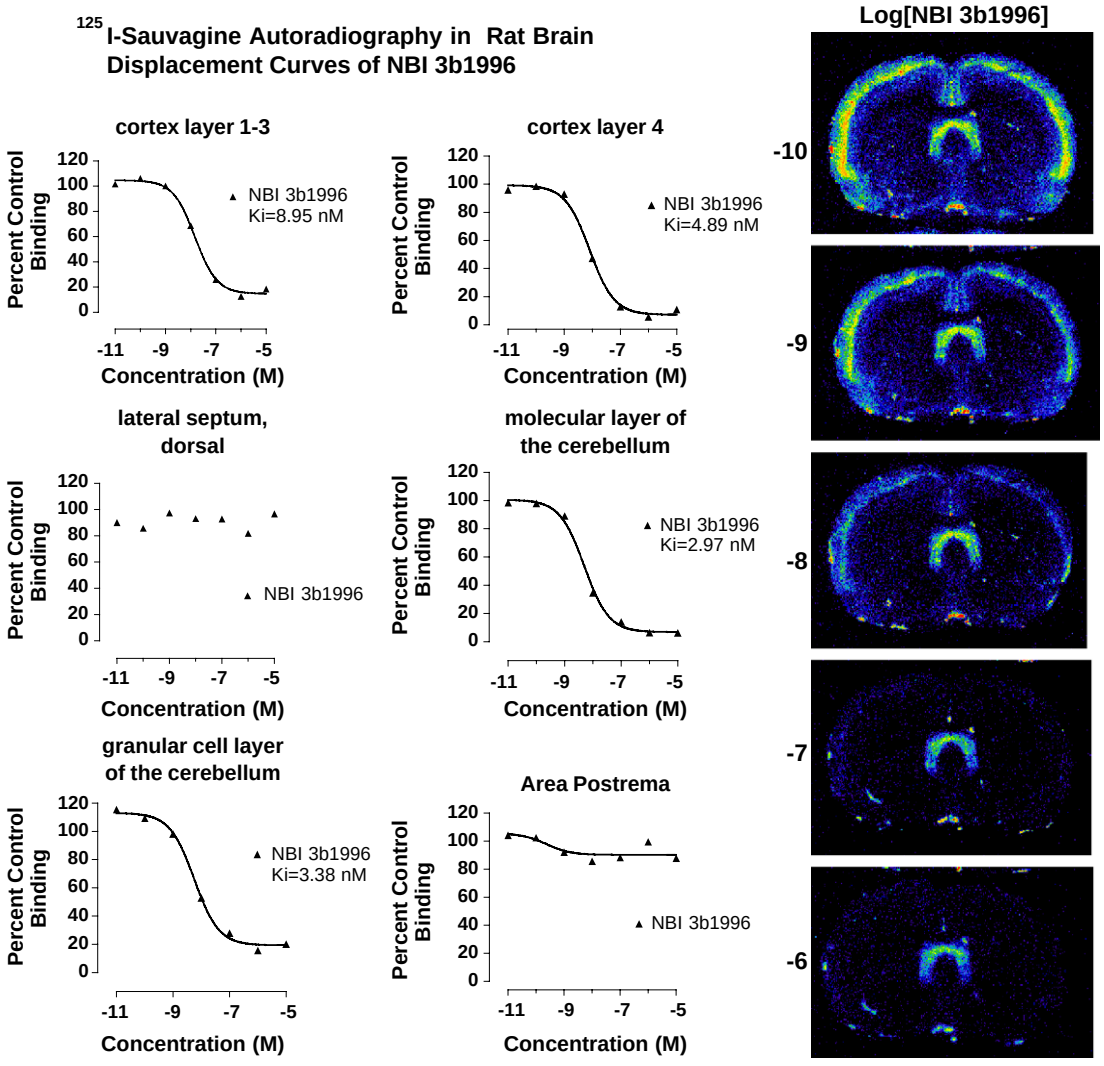

Fig. 2. Autoradiographic evaluation of NBI3b1996 potency and selectivity for ^{125}I-sauvagine binding to CRF_1 and CRF_2 receptors in the rat brain. Sections of rat brain were incubated with ^{125}I-sauvagine in the absence and presence of various concentrations of NBI3b1996. The labeled sections were exposed to film with radioactive standards and quantitated. The dose–response curves are seen in the left hand side of the figure while representative autoradiograms are seen on the right. In general, NBI3b1996 potently inhibited binding in CRF_1 containing brain regions such as the cerebral cortex but exhibited little affinity for CRF_2 containing regions such as the lateral septum (n=4).

were incubated in the presence of ^{125}I-sauvagine with various concentrations of NBI3b1996. The resulting autoradiograms were quantitated using image analysis and radioactive standards. In these studies, NBI3b1996 was found to potently inhibit ^{125}I-sauvagine binding to the cerebral cortex and the cerebellum. On the other hand, very little inhibition was seen in the lateral dorsal septum, an area predominantly containing the CRF_2 receptor. In subsequent experiments, the compound is found to inhibit ^{125}I-sauvagine binding to cerebellar homogenates with a K_i of approximately 3 nM (data not shown).

3.2. Assessment of in vivo potency of NBI3b1996 using ex vivo binding

When the in vitro potency and selectivity of NBI3b1996 was established, the in vivo potency of this compound was evaluated using ex vivo binding (Fig. 3). In these experiments, rats received the compound by the i.p. route of administration, were sacrificed and the cerebella removed. The tissue was homogenized and subjected to ^{125}I-sauvagine binding. Compound within the tissue inhibits the binding of the radioligand to CRF_1 receptors. The CRF_1 receptor antagonist produced a dose-dependent decrease in ^{125}I-sauvagine binding with an estimated ED_{50} of 6 mg/kg. Significant inhibition was achieved at doses of 10, 30 and 100 mg/kg.

3.3. Assessment of the effects of NBI3b1996 in social interaction

To determine which dose of the CRF_1 receptor antagonist was able to block the anxiogenic effects of Urocortin (100 fmol) a dose–response study was designed. The results indicated that animals that received a 3 mg/kg dose of the CRF_1 receptor antagonist still showed an increase in anxiety-like behavior with a significant decrease in Social Interaction time as compared to their baseline scores (Fig. 4A: t=2.902; P=0.03). In addition, animals in group two which received the 5 mg/kg dose of the antagonist also still displayed anxiogenic-like behavior (Fig. 4B: t=2.994; P=0.03), however, rats treated with the highest dose of the antagonist (Fig. 4C; 10 mg/kg) were observed to have no difference in Social Interaction time between the two conditions. Control rats treated with vehicle prior to Urocortin showed a significant decrease in Social interaction time as compared to baseline (Fig. 4D: t=5.821; P=0.0001). Animals treated with the compound alone showed no significant difference in social interactions times at any dose tested (Fig. 5).

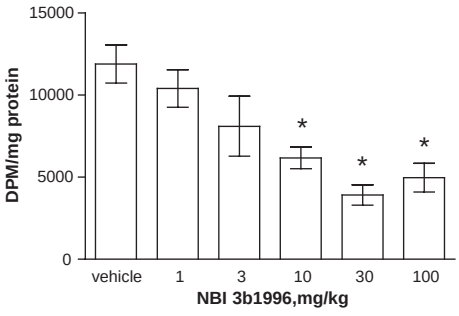

Fig. 3. Inhibition of ^{125}I-sauvagine by NBI3b1996 ex vivo. Rats were administered NBI3b1996 by the i.p. route and sacrificed 1 h later. ^{125}I-sauvagine binding to cerebellar homogenates was assessed using a centrifugation assay. *P<0.05 ED_{50}=6 mg/kg, i.p.

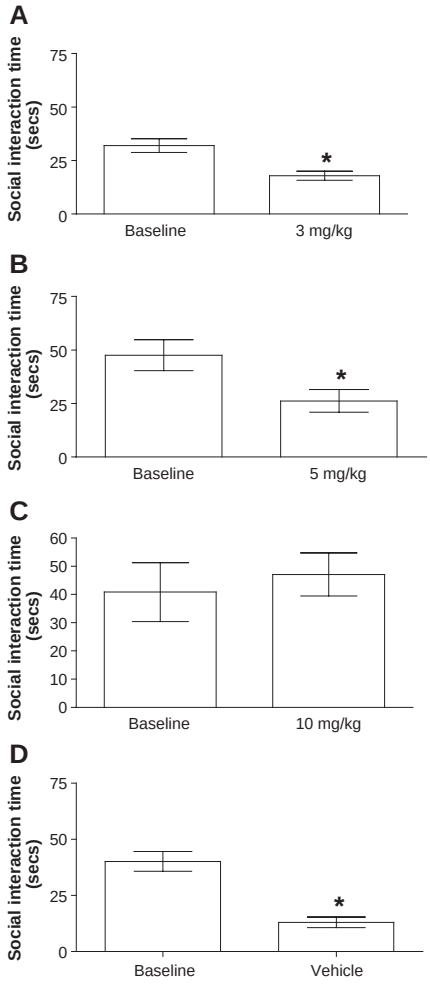

Fig. 4. Changes in social interaction time in animals administered vehicle i.p.+vehicle into the basolateral nucleus of the amygdala (baseline) and (A) CRF_1 receptor antagonist (3 mg/kg i.p.)+Urocortin (100 fmol) into the basolateral nucleus of the amygdala; (B) CRF_1 receptor antagonist (5 mg/kg i.p.)+Urocortin (100 fmol) into the basolateral nucleus of the amygdala; (C) CRF_1 receptor antagonist (10 mg/kg i.p.)+Urocortin (100 fmol) into the basolateral nucleus of the amygdala and (D) vehicle i.p.+Urocortin (100 fmol). *Significantly different from baseline, P<0.05 (n=4).

3.4. The effects of NBI3b1996 on restraint stress-induced reductions in SI

Following baseline social interaction scoring, animals were subjected to three consecutive days of restraint stress

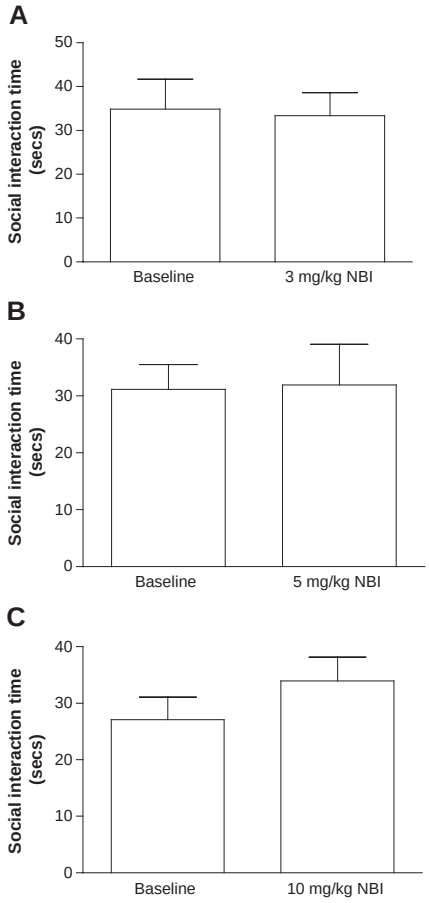

Fig. 5. The effect of NBI3b1996 on baseline social interaction times observed in the Social Interaction test. NBI3b1996 was administered at doses of 3 (A), 5 (B) and 10 (C) mg/kg, i.p. No statistically significant effect was seen at any of the doses tested.

and allowed to recover for 3 days. On the testing day, the animals were administered the test compound or vehicle and 30 min later were subjected to 30 min of restraint stress. The animals were then placed into the Social Interaction box with a novel partner rat and the Social Interaction time was determined. The 30 min restraint produced a marked reduction in the Social Interaction time (Fig. 6). Administration of NBI3b1996 produced a dose-dependent restoration of Social Interaction times to baseline values [$F(4,35)=21.88$; $P=0.0001$].

4. Discussion

CRF has been shown to have an important role in initiating the biological events known to occur during the stress response (Vale et al., 1981). In addition, central administration of CRF produces anxiogenic-like behavior in several behavioral tests such as open field (Britton et al., 1982; Liang and Lee, 1988; Takahashi et al., 1989), elevated plus maze (Baldwin et al., 1991), conflict test (Britton et al., 1985), social interaction (Dunn and File, 1987), acoustic startle (Swerdlow et al., 1986) and conditioned fear paradigm (Cole and Koob, 1988). A relatively new member of the CRF peptide family, Urocortin was recently cloned and characterized (Spina et al., 1996). While CRF has higher affinity for CRF_1 compared to CRF_2, Urocortin has high affinity for both receptors in vitro. Similar to CRF, Urocortin induces anxiety-like behaviors in rodents (Moreau et al., 1997). Because of the mixed agonist activity of Urocortin, we sought to understand the contribution of the CRF_1 receptor to the anxiety-like behaviors induced by this peptide.

The basolateral nucleus of the amygdala, a temporal lobe structure that is critical for the emotional response to fear, is an important brain structure in the stress/anxiety response (Sajdyk and Shekhar, 1997a,b; Sanders et al., 1995; Sanders and Shekhar, 1991, 1995a,b). This nucleus has been proposed to function as an integration center between other nuclei of the amygdala to elicit the appropriate response to fearful stimuli (Campeau and Davis, 1995; LeDoux, 2003). In our previous studies, we found that CRF and Urocortin administration into the basolateral nucleus of the amygdala results in a robust, dose-dependent decrease in social interaction times (Sajdyk et al., 1999). Previous studies have shown that the CRF_1 receptor is the predominant subtype in the basolateral nucleus of the amygdala (Reul and Holsboer, 2002). Consistent with the relative in vitro affinities of Urocortin and CRF for the CRF_1 receptor, Urocortin was considerably more potent than CRF in producing this response. The reductions in social interaction times were antagonized by prior infusion of the nonselective antagonist, astressin (Sajdyk and Gehlert, 2000). Furthermore, repetitive infusion of subthreshold doses of Urocortin into the basolateral nucleus of the amygdala primed anxiety-like behaviors. As a result, the animals became behaviorally and physiologically responsive to lactate, in a manner similar to that observed with patients with panic disorder (Sajdyk et al., 1999). Repetitive infusion for 5 days resulted in persistent anxiety-like behaviors and profound changes in the neuronal activity of the basolateral nucleus of the amygdala (Rainnie et al., 2004). Overall, the results are consistent with an important role for the CRF system, not

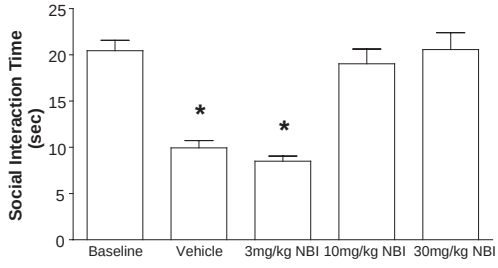

Fig. 6. The effect of NBI3b1996 on social interaction behavior following repeated restraint stress. Rats were evaluated for their baseline social interaction time and then subjected to three consecutive days of restraint stress and allowed to recover for 72 h. One hour prior to testing, animals were administered compound or vehicle and 30 min later subjected to 30 min of restraint stress. The social interaction time was evaluated immediately thereafter. *$P<0.001$ ($n=8$).

only in the behavioral manifestation of mood disorders, but potentially in their progression as well.

The CRF peptide family is believed to produce effects via two G-protein coupled receptors, CRF_1 and CRF_2 (Dautzenberg and Hauger, 2002). Both these receptors are present in the central nervous system with unique distributions observed in several species (Sanchez et al., 1999). Using autoradiographic techniques, high levels of CRF_1 binding sites were localized to the amygdala (DeSouza et al., 1985) and the greatest density of CRF_1 mRNA was localized to the basolateral nucleus of the amygdala (Chalmers et al., 1995). In previous studies, the CRF_1 receptor was implicated in the anxiety-like responses observed in animal models after intracerebroventricular CRF infusion (Risbrough et al., 2003). In the present study, we sought to understand that role of the CRF_1 receptor subtype in the anxiety-like responses observed in the Social Interaction test after intra-basolateral nucleus of the amygdala infusion of Urocortin. To accomplish this, we assessed the potency and selectivity of the nonpeptide CRF_1 receptor antagonist NBI3b1996 (Chen et al., 1996). It potently inhibited the binding of ^{125}I-sauvagine to the cerebral cortex where the CRF_1 receptor predominates while having little effect on binding to the lateral septum where the CRF_2 receptor predominates. Therefore, this antagonist appears to be highly selective for the CRF_1 receptor subtype in rat brain. To assess the potency of the CRF_1 receptor antagonist in vivo, we utilized the technique of ex vivo binding. In these studies, rats were administered the compound and sacrificed 1 h later. Binding of ^{125}I-sauvagine to the cerebellum was assessed ex vivo using a centrifugation assay. The rat cerebellum contains primarily the CRF_1 receptor subtype and, therefore, could be used to assess the potency of the compound for the CRF_1 receptor in vivo. Using this technique, we estimated an ED_{50} of 6 mg/kg when administered by the i.p. route. Therefore, we demonstrated that NBI3b1996 was a potent and selective CRF_1 receptor antagonist in vitro and in vivo with suitable potency to be used for behavioral studies.

To determine the effects of the compound on Urocortin-induced anxiety-like behaviors in the social interaction test, we administered the CRF_1 receptor antagonist 30 min prior to infusing Urocortin into the basolateral nucleus of the amygdala. The CRF_1 receptor antagonist dose-dependently antagonized the effects of Urocortin with an estimated ED_{50} of 6 mg/kg by the i.p. route. The compound had little effect on social interaction in the absence of Urocortin infusion suggesting that little activation of CRF_1 occurs under the conditions of the social interaction test. In addition, the compound had similar potency in both the ex vivo binding and social interaction tests providing evidence that the compound antagonized the Urocortin effect at doses consistent with its interaction with the CRF_1 receptor. We then explored the effects of the CRF_1 receptor antagonist on stress-induced reductions in social interaction. In this experiment, the animals were subjected to three sessions of a 30-min restraint stress. After 3 days without restrain stress, the animals were dosed with compound or vehicle and subjected to an additional session of restraint stress. Following this paradigm, the animals exhibited a marked reduction in social interaction times. The CRF_1 receptor antagonist dose-dependently reversed the effect of stress on social interaction. Therefore, the CRF_1 receptor plays an important role in the effects of stress on this behavioral paradigm.

Results from the present study constitute additional evidence that the CRF_1 receptor mediates the anxiogenic actions of CRF and Urocortin in the amygdala. The antagonism of the Urocortin-induced reduction in social interaction occurred at similar doses to those required for central CRF_1 receptor occupancy. This study, along with many others, supports the potential for CRF_1 receptor antagonist in the treatment of anxiety and depression. Previously, nonpeptide CRF_1 receptor antagonists were reported to exhibit anxiolytic-like actions in the animal models of anxiety disorders (for review, see Kehne and De Lombaert, 2002). Interestingly, several compounds have been reported to increase social interaction behaviors in the social interaction test. Millan et al. (2001) reported that the CRF antagonists butyl-[2,5-dimethyl-7-(2,4,6-trimethyl-phenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]e thylamine (CP 154526) and *N*-(2-chloro-4,6-dimethylphenyl)-1-[1-methoxymethyl-(2-methoxyethyl]-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine mesylate (DMP 695) produced significant increases in social interaction at doses of 2.5 and 40, respectively. In the present study, however, we did not observe an effect of NBI3b1996 on social interaction in the absence of Urocortin infusion. The apparent discrepancy may lie in technical differences in how the social interaction experiments were conducted. For instance, in our experimental paradigm, we treated only one rat and monitored the treated rats' ability to initiate social interaction with an untreated animal. In the other studies, both rats were treated with the test compound and monitored. Further understanding of the differences between these methods will require carefully controlled comparison studies using the same compounds under the same environmental conditions.

CRF appears to be one of the more important neurotransmitters/neuromodulators in the amygdala. A high density of CRF, but not Urocortin immunoreactivity is found within the amygdala (Morin et al., 1999) and, using microdialysis techniques, restraint stress produced increases in the release of CRF from the amygdala (Hand et al., 2002). Environmental stressors have been shown to upregulate the components of the CRF including the receptors and peptide (for review, see Bakshi and Kalin, 2000). Therefore, plasticity in the CRF system may be an important contributor to its role in psychiatric disorders. Beyond its anxiogenic role, CRF and Urocortin also appear to be important in the formation and retention of emotional memory via actions within the basolateral nucleus of the amygdala (Roozendaal et al., 2002).

Repeated infusions of subthreshold doses of Urocortin or CRF into the rat basolateral nucleus of the amygdala result in lactate sensitivity, a hallmark of panic disorder in human patients (Sajdyk et al., 1999). In addition to established anxiogenic actions, CRF may play an important role in sensitizing the amygdala to environmental stressors. Thus, antagonism of central CRF_1 receptors may represent a mechanism that can prevent the progression of psychiatric disorders as a consequence of emotional, physical or chemical stressors.

In conclusion, the selective nonpeptide CRF_1 receptor antagonist NBI3b1996 produced a dose-dependent inhibition of intra-basolateral nucleus of the amygdala Urocortin infusion-induced reductions in social interaction. Therefore, the CRF_1 receptor appears to be the predominant receptor subtype mediating the anxiogenic-like effects of Urocortin in the basolateral nucleus of the amygdala. The CRF antagonist did not produce an effect on social interaction under baseline conditions but reversed stress-induced reductions in SI. These results provide further evidence that selective CRF_1 receptor antagonists may have clinical utility as anxiolytic agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2004.12.030.

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