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Research report

Repeated intracerebral teneurin C-terminal associated peptide (TCAP)-1 injections produce enduring changes in behavioral responses to corticotropin-releasing factor (CRF) in rat models of anxiety

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Abstract

The teneurin C-terminal associated peptides (TCAP) are a recently discovered family of peptides encoded by a bioactive neuropeptide-like gene sequence found at the carboxy terminus of the teneurin transmembrane proteins. TCAP is structurally related to the corticotropin-releasing factor (CRF) family of peptides. Synthetic TCAP-3 and TCAP-1 are active in vitro in stimulating cAMP and proliferation in neuronal lines. TCAP-1 mRNA is expressed in limbic brain regions and modulates acoustic startle behavior in rats when injected into the basolateral amygdala. In the current study, TCAP-1 was administered into the cerebral ventricles once per day for 5 days to rats. At 1–3 weeks after the last TCAP-1 treatment, the rats were tested in the elevated plus maze, open field test, or the acoustic startle test, with or without an acute CRF injection 30 min prior to the test. The results show a difference in behavioral response between TCAP-treated and saline-treated rats, but only when an acute CRF challenge is delivered prior to testing. In the plus maze and open field tests, acute CRF effects were enhanced by prior TCAP-1 treatment, whereas in the acoustic startle test, the acute CRF effects were diminished by prior TCAP-1 administration.

Keywords: Teneurin; Corticotropin-releasing factor; Acoustic startle; Plus maze; Open field

1. Introduction

The teneurin C-terminal associated peptides (TCAP) are a recently discovered family of neuropeptides [13,23]. This peptide is encoded by a bioactive neuropeptide-like sequence present at the carboxy terminus of the teneurin transmembrane proteins. The teneurins are a family of four highly conserved proteins that belong to a novel class of signalling molecules [22], each of which possesses the TCAP sequence at the C-terminus (TCAP1-4). The TCAP sequence encodes a peptide 40 or 41 amino acids long, and is flanked by a cleavage motif on the amino terminus and an amidation motif on the carboxy terminus, characteristic of bioactive peptides.

We have previously reported that the mRNA containing the TCAP-1 sequence is expressed in key forebrain and limbic regions of the rat brain involved in regulating emotion and responses to stress [23]. In vitro, TCAP-1 induces a dose-dependent change in cyclic AMP accumulation and MTT activity in immortalized mouse neurons. We have also shown that synthetic rainbow trout TCAP-3 increases cAMP, stimulates proliferation, and regulates teneurin-1 gene expression in a neuronal cell line [13]. TCAP-1 increases neurite outgrowth in part by inducing β-tubulin transcription and translation in immortalized mouse hypothalamic neurons and rat hippocampal primary cultures [1]. In addition, TCAP-1 alters the acoustic startle response in rats when injected into the basolateral amygdala or into the cerebral ventricles, suggesting that it may play a role in regulating emotional behavior [23].

The TCAP sequence has some primary structure similarity to that of the corticotropin-releasing factor (CRF) family of peptides. The TCAP peptides show 13–21% identity at the amino

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acid level to the CRF family of peptides [23]. In fact, the TCAP sequence was discovered during a low-stringency screen for paralogous genes to the CRF family [13]. The CRF family of peptides is well known for its involvement in behavioral and physiological responses to stress [5,12]. Dysregulation of the CRF system is widely believed to be involved in the pathophysiology of psychiatric disorders [10,18] and drug dependence [3,6]. Furthermore, pharmacological manipulations of the CRF system may be important in their treatment [11].

Previously, we showed that repeated injections of TCAP had long-lasting effects on acoustic startle behavior [23]. In the current study, we examine the effects of repeated intracerebroventricular (ICV) injection of synthetic TCAP-1 to rats in three different animal models of anxiety: the elevated plus maze, the open field test, and the acoustic startle test. In addition, we examined the interaction of this regimen of TCAP-1 treatment with an acute ICV injection of CRF, a known stressor, to study the potential interactions of TCAP with CRF, given their structural similarity.

2. Methods

2.1. Animals

Male Wistar rats weighing 250–300 g were obtained from Charles River Laboratories (Montreal, Canada). Rats were singly housed in Plexiglas shoebox cages under standard laboratory conditions (food and water available ad libitum, temperature 21 ± 1 °C, lights on at 0700, off at 1900). All testing took place between 0900 and 1700. Rats were given 1 week to acclimatize to the laboratory before surgery. All procedures were approved by the local animal care committee and were in accordance with the Canadian Council on Animal Care.

2.2. Surgery

The rats were anaesthetized with 1–4% isoflurane/oxygen gaseous mix and fit into a stereotaxic apparatus. A mid-line incision was made along the top of the head, exposing bregma; the cannula was implanted into the right lateral ventricle using the following flat-skull stereotaxic coordinates: AP −1.0 mm, ML +/−1.4 mm and DV −2.7 mm from dura [14]. A 22-gauge guide cannula (Plastics One, Roanoke, VA, USA) was fixed to the skull with dental acrylic and jewellers' screws. Dummy guides were placed into the cannulae to prevent blockage and to prevent debris from entering the brain. The animal was kept warm under a lamp until regaining consciousness.

2.3. Drugs

Synthetic mouse 10^{-4} M TCAP-1 was synthesized and prepared for injection as previously described [23]. CRF (Sigma–Aldrich, Oakville, ON) was dissolved in saline at a concentration of 1 μg/μl.

There were 6 treatment groups for the elevated plus maze and open field: TCAP + CRF (0 μg), TCAP + CRF (1 μg), TCAP + CRF (3 μg), SAL + CRF (0 μg), SAL + CRF (1 μg), and SAL + CRF (3 μg). There were 4 treatment groups for acoustic startle: TCAP + CRF (0 μg), TCAP + CRF (3 μg), SAL + CRF (0 μg), and SAL + CRF (3 μg).

2.4. Injections

Beginning 1 week after surgery, the rats used for elevated plus maze and open field tests were injected (ICV) once daily with 300 pmol (3 μl of 10^{-4} M) TCAP-1 or saline (for control) for 5 days. The injection was delivered at the speed of 2 μl/min by a syringe pump (Razel Scientific Instrument Inc., Stamford, USA). Drugs were infused using a 28-gauge stainless steel injector that extended 1 mm below the cannula guide tip. The injector was left in place for a period of 60 s following infusion to prevent backflow up the cannulae.

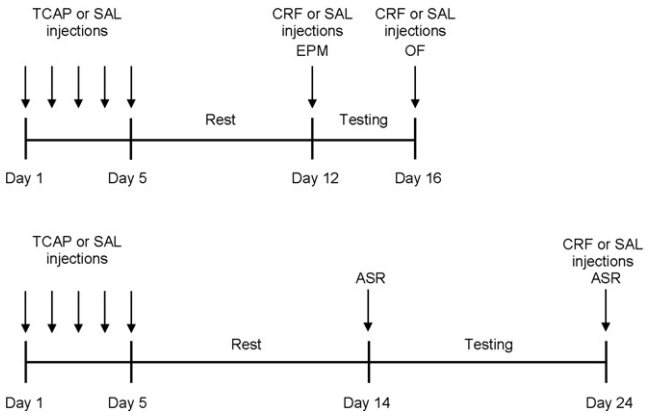

Fig. 1. (Top) Treatment and experiment timeline for the elevated plus maze and open field tests. (Bottom) Treatment and experimental timeline for the acoustic startle test.

Details of the injection schedule and testing procedure are depicted in Fig. 1. Following the five injection days, the rats used for elevated plus maze and open field were not injected or tested for a period of 7 days (Fig. 1, top). On the 7th day after cessation of injections (day 12), the rats were tested on the elevated plus maze. On the 11th day after the injections (day 16), the rats were tested in the open field.

A separate group of rats was used for the startle testing. For the rats tested in the acoustic startle test, there were 5 days of TCAP-1 injections, followed by 9 days without treatment, and were tested as shown in Fig. 1 (bottom).

Acute ICV injections of CRF (0, 1, 3 μg) were administered by syringe pump 30 min prior to behavioral testing in the case of the plus maze and open field tests, and immediately before testing in the case of the startle test. Only the 0 and 3 μg doses of CRF were used in the startle test.

2.5. Elevated plus maze

The elevated plus maze test was conducted using a standard plus maze apparatus elevated 65 cm above the floor, consisting of two enclosed arms (50 cm × 10 cm) and two open arms (50 cm × 10 cm). The enclosed arms had walls 40 cm high. The four arms were joined at the center by a 10 cm square platform. The apparatus was painted flat black and was illuminated by a dim red light (25 W bulb). The maze was cleaned with mild soap and ethanol between tests.

On the 7th day after the end of the TCAP injections, the rats were injected ICV with CRF (0, 1 or 3 μg). Thirty minutes later, the animals were placed individually in the center of the plus maze facing one of the open arms. Behavior was recorded for 5 min by a digital camera suspended above the maze. This signal was tracked, quantified, and analyzed using an Ethovision® video tracking system (Noldus Information Technology, Utrecht, Netherlands).

2.6. Open field test

On the 11th day after the end of the TCAP injections (4 days after the plus maze test), the rats were tested for locomotor activity in the open field test. The apparatus consisted of a 50 cm × 50 cm arena with 40 cm high walls made of black particle board. A 30 cm × 30 cm square in the center of the open field was defined as the center zone for data analysis.

Animals received injections of either 0, 1, or 3 μg CRF (ICV) 30 min prior to testing. At test time, the rats were gently placed in the apparatus at the start of the session, and their movement was recorded by a digital camera mounted over the apparatus. This signal was tracked, quantified, and analyzed using an Ethovision® video tracking system (Noldus Information Technology, Utrecht, Netherlands). The apparatus was illuminated by a dim red light (25 W bulb). All tests were 60 min in duration.

2.7. Acoustic startle

The rats ($n = 16$) were tested for acoustic startle responses in sound-attenuated acoustic startle reflex (ASR) chambers (Med Associates, Inc., St. Albans, VT, grid rod cage measuring 7.5 in. × 3.6 in. × 4.2 in.). Background noise was set at 70 dB of white noise. One week following surgical implantation of cannulae into the lateral ventricles, the rats were given a session of 60 presentations of an acoustic stimulus (120 dB) with a 50–70 s inter-stimulus interval. Each stimulus was presented for a period of 30 ms, at a frequency of 5000 Hz. This data was used to match the rats into two treatment groups on the basis of baseline startle responses. The rats then received 5 consecutive days of ICV injections of either TCAP or saline as described above. On day 14 after the injections, the rats were tested in the acoustic startle test. Rats received a 5-min acclimation period followed by 60 presentations of 120 dB stimuli with an inter-stimulus interval of 50–70 s. Startle responses were measured and recorded by the proprietary software associated with the startle apparatus (Med Associates, Startle Reflex version 4.10). Analysis of the data indicated there was no difference between the groups. Therefore, on day 24 after the TCAP injections, the rats were tested with an acute injection of CRF. On the test day, the rats received a 25 min baseline startle session, following which they were removed from the startle chambers, and injected with 3 μg of CRF (3 μl) and then were immediately placed back in the startle apparatus for a further 75 presentations of startle stimuli using the same parameters as above. At the end of behavioral testing, the brains were examined for cannulae placements.

2.8. Data analysis

Behavior in the plus maze and open field tests was quantified using Ethovision® proprietary software. Startle data was collected using MedAssociates proprietary software. Raw data from both of these programs was then transferred to Microsoft Excel for sorting and analysis. Statistical analysis of all data was carried out with GraphPad Prism version 4.0 as described below. Only data from rats with confirmed cannula placements were used.

3. Results

3.1. Elevated plus maze

Data for TCAP + CRF (0 μg), TCAP + CRF (1 μg), TCAP + CRF (3 μg) ($n = 11, 8, 8$, respectively), and SAL + CRF (0 μg), SAL + CRF (1 μg), SAL + CRF (3 μg) ($n = 10, 13, 7$, respectively), were obtained. TCAP group data was converted to a percent of control for the corresponding saline group at each dose of CRF (0, 1, 3 μg). Percent open arm time was calculated as [open arm time/(total arm time) × 100]; percent open arm entries was calculated as [open arm entries/(total arm entries) × 100]. These values were analyzed by a one-sample t-test against a hypothetical mean of 100% (Fig. 2, top).

The TCAP + CRF (1 μg) group differed significantly from the SAL + CRF (1 μg) control ($t = 2.718$, $p = 0.0299$) for percent of open arm time, with a mean 38% less time spent on the open arms than that of control animals. Similarly, the TCAP + CRF (1 μg) group significantly differed from its saline control in open arm entries ($t = 4.986$, $p = 0.0016$), with a mean 36% less entries than the SAL + CRF (1 μg) group (Fig. 2, bottom).

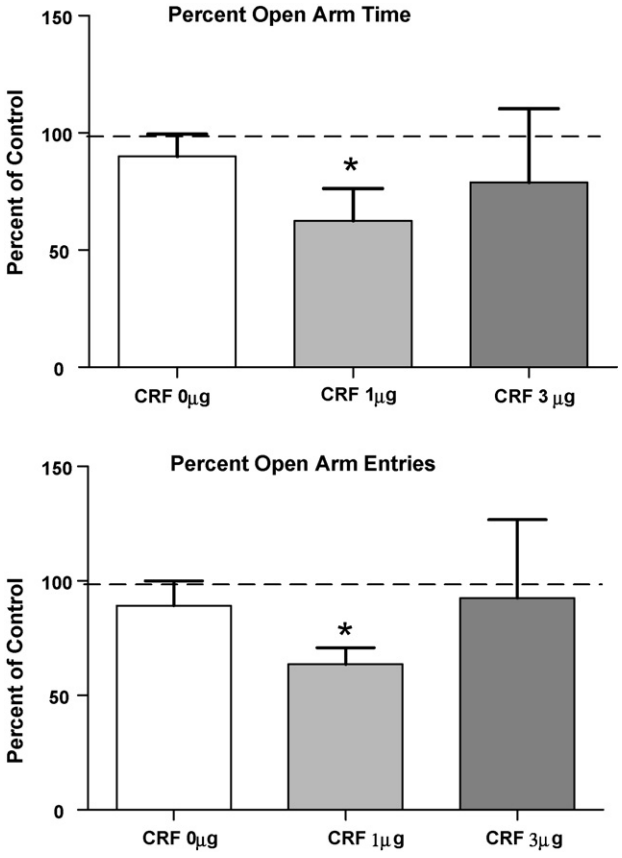

Fig. 2. Percent of control data for the elevated plus maze test. Top panel shows percent open arm time, and bottom panel shows percent open arm entries for the three treatment groups. Percent open arm time and entries was significantly reduced in the TCAP + CRF (1 μg) group relative to the SAL + CRF (1 μg) group, indicating that TCAP pre-treatment potentiated the effects of the acute injection of 1 μg CRF. (*$p < 0.05$).

3.2. Open field test

Data for TCAP + CRF (0 μg), TCAP + CRF (1 μg), TCAP + CRF (3 μg) ($n = 7, 7, 6$, respectively), and SAL + CRF (0 μg), SAL + CRF (1 μg), SAL + CRF (3 μg) ($n = 7, 6, 7$, respectively) were obtained. TCAP group data was converted to a percent control for the corresponding saline group at each dose of CRF (0, 1, 3 μg).

Several measures of locomotor behavior were examined: total distance traveled, distance traveled in the center zone, time spent in the center zone, and entries into the center zone. For distance moved, a 2 cm minimum distance was required to be scored as genuine displacement. Data is presented in Fig. 3.

The TCAP + CRF (3 μg) group differed significantly from control in total distance traveled ($t = 4.596$, $p = 0.0059$), with a mean 28% less distance traveled than the SAL + CRF (3 μg) group. Both the TCAP + CRF (1 μg) and the TCAP + CRF (3 μg) groups differed from their controls in center distance traveled ($t = 2.688$, $p = 0.0371$; $t = 2.523$, $p = 0.053$, respectively), although the TCAP + CRF (3 μg) group did not differ significantly from its control at the 0.05 level. In a measure of center entries, the TCAP + CRF (3 μg) group significantly differed from control ($t = 5.263$, $p = 0.0033$) with a mean of 51% of control values. Finally, centre time differed significantly between both TCAP + CRF (1 μg) and TCAP + CRF (3 μg) from its controls ($t = 3.339$, $p = 0.0156$; $t = 3.9$, $p = 0.0114$, respectively). The TCAP + CRF (1 μg) group spent 52% less time in the center of the open field over the SAL + CRF (1 μg) controls, while the

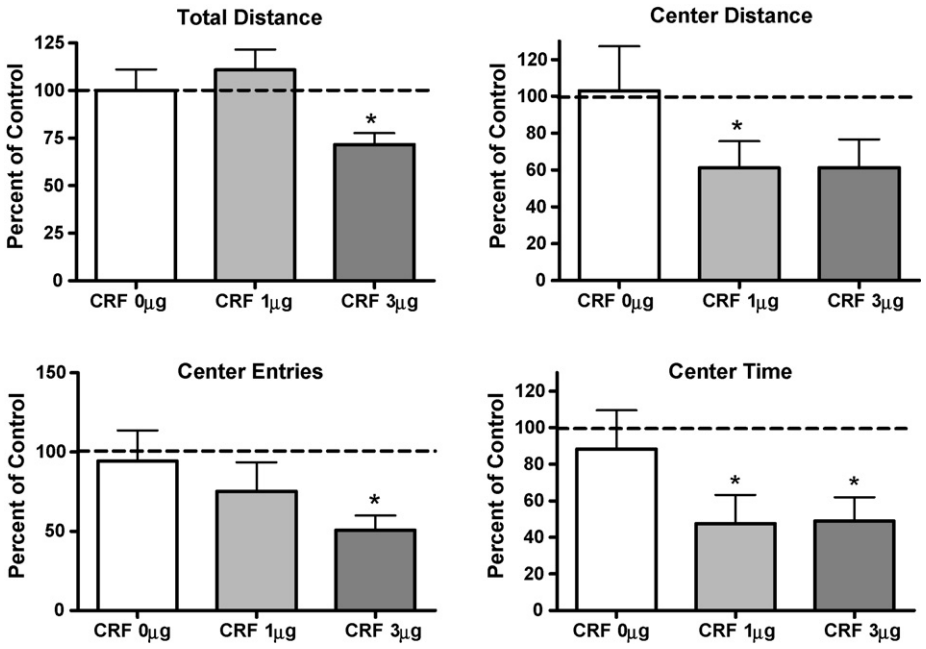

Fig. 3. Percent of control data for the open field test. Rats treated with TCAP + CRF had significantly lower total distance, and center distance, time, and entries compared with their saline pre-treated controls. (*$p < 0.05$).

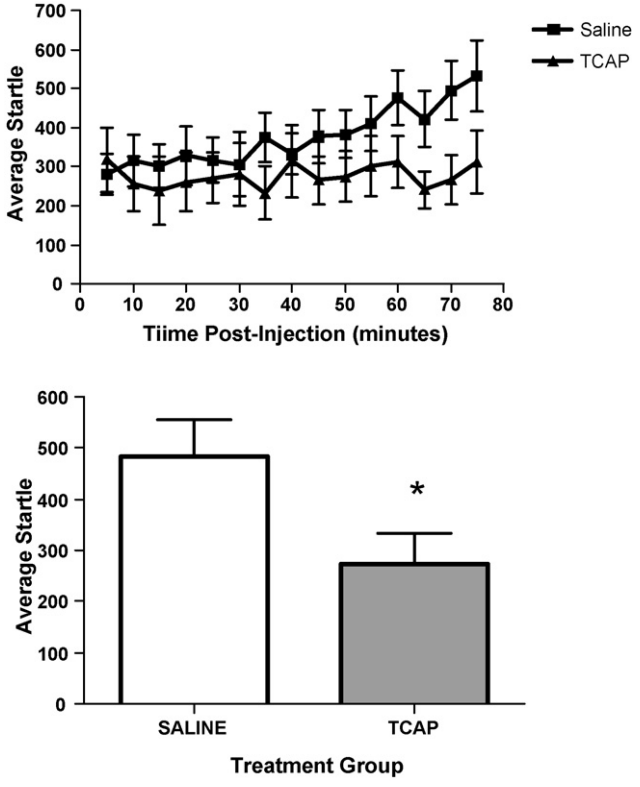

Fig. 4. (Top) Average startle for rats previously treated for 5 days with either vehicle or TCAP. Data is shown in 5 min bins over the 75 min post-injection period. All rats received an ICV injection of CRF (3 μg) immediately before testing. (Bottom) The startle scores for the last 15 min of the test were averaged for each rat. (*$p < 0.05$).

TCAP + CRF (3 μg) group spent 49% less time in the center of the open field over the SAL + CRF (3 μg) controls.

3.3. Acoustic startle

Data from 16 rats were included (TCAP, $n = 8$; Saline, $n = 8$). The startle data was analyzed using a two-factor repeated ANOVA with "time" and "drug treatment" as factors. There was a significant interaction of time and drug treatment ($F = 1.795$, $p = 0.0415$), and time ($F = 2.326$, $p = 0.0055$) (Fig. 4, top). Given the significance of these findings, further analysis of the data was carried out on the last 15 min of the test only. This time was chosen because the effects of CRF on startle responses are not evident until approximately 60 min post-injection [8,20]. An average startle score was calculated for each rat, and these values were analyzed with an unpaired *t*-test. This analysis revealed a significant difference between the mean startle values for the two groups ($t = 2.232$, $p = 0.0424$) (Fig. 4, bottom).

4. Discussion

The data presented in this study indicate that repeated ICV TCAP-1 administration has long-lasting effects on behavioral responses to CRF in rat models of anxiety. It is important to note that by itself, TCAP-1 had no effect on performance in the startle, elevated plus maze, or open field tests. Thus, there were no gross motor effects, or effects on baseline levels of anxiety or exploratory behavior. However, TCAP-1 did affect behavior in the presence of a CRF challenge. This indicates that TCAP-1 may be acting as a behavioral modulator. This finding is supported by our previous work [23], which showed that an acute TCAP-1 injection into the basolateral amygdala of rats

had differential effects on the startle response depending upon the rat's baseline reactivity.

The elevated plus maze has been used extensively as a model of anxiety [4,16]. Anxiolytic manipulations will generally increase open arm time and entries, whereas anxiogenic treatments generally decrease them. In the present plus maze test, the TCAP-treated rats showed a potentiated effect of CRF (1 μg) treatment. Rats treated with TCAP + CRF (1 μg) showed decreased percent open arm time and entries relative to rats treated with SAL + CRF (1 μg). This is consistent with an anxiogenic effect of CRF, and with literature reports that the percent time spent in the open arms of the elevated plus maze is significantly reduced in rats 30 min after an ICV infusion of 1 μg CRF [17].

The open field test involves placing a rat in an arena, and measuring the distance travelled as well as the time spent in different sectors of the arena. It measures locomotor activity, exploration and response to a novel, aversive environment. Rats will generally avoid the center portion of the chamber, and anxiolytic manipulations will increase time spent, as well as distance travelled, in the center portion of the chamber. In the open field test, TCAP pre-treatment potentiated the effects of an acute injection of CRF. Rats pre-treated with TCAP and acutely treated with CRF (1 or 3 μg) showed decreased total locomotion, as well as time, entries and distance travelled in the center zone, relative to their SAL pre-treated controls. Only under conditions of a CRF challenge did differences between the TCAP-treated and SAL-treated rats appear. The CRF effect of decreased total locomotion and decreased center activity is consistent with an anxiogenic effect of CRF. Although CRF can have behavioral activating effects in rats, CRF-treated rats show decreased locomotion and rearing when tested in a novel, aversive environment [2,7,19]. Thus, TCAP pre-treatment appears to have potentiated the effects of the acute CRF injection.

In the startle test, in contrast to the plus maze and open field tests, TCAP pre-treatment did not enhance the effects of an acute CRF injection, but rather, *prevented* the CRF-induced increase in startle. However, following CRF administration, the saline-treated rats showed the characteristic increase in startle response one hour after injection, whereas the TCAP-treated rats did not. Using a similar design, Swerdlow and Britton [20] found that CRF injected ICV at a dose of 1 μg increased acoustic startle responses at 60 min, but not immediately, after injection. Liang et al. [8] also reported this delayed effect, which is consistent with our results.

Although it appears contradictory that TCAP would potentiate the effects of CRF in some behavioral tests (plus maze, open field) and block the effects of CRF in another (acoustic startle), paradoxical observations among behavioral paradigms is not unprecedented in the literature. Other studies have noted anxiolytic-like effects in some behavioral tests but not in others. For example, Roman Low Avoidance (RLA) rats show significant fear potentiation in the acoustic startle test, as compared with Roman High Avoidance (RHA) rats, but RLA have similar or even less anxiety in the open field and plus maze tests than RHA [24]. Alphaxalone, a steroid anaesthetic that acts on the GABA/benzodiazepine receptor, decreases CRF-enhanced startle without affecting CRF-enhanced locomotor activity [20]. Thus, an anxiolytic response in one anxiety-related task does not necessarily mean that an anxiolytic response will be seen in other anxiety-related tasks.

It is also interesting to note that the tests of exploration (open field, plus maze) show consistent behavioral effects with each other, whereas the test of a reflexive fear response (startle) shows a different effect. The demands of different behavioral tasks not only assess different types of fear or anxiety behavior, but will likely recruit different brain regions. For example, the amygdala is the critical substrate for CRF-enhanced startle [9], but not for CRF-enhanced locomotion [21]. We have previously reported that TCAP-1 mRNA is highly expressed in the basolateral and central nuclei of the amygdala, and that acute TCAP-1 injections into the basolateral amygdala have modulatory effects on acoustic startle that depend upon the baseline reactivity of the rat [23]. CRF may differentially regulate the nuclei of the basolateral and central amygdala due to heterogeneity of its receptors in these regions [15]. In addition, other brain regions such as the hippocampus may be more important in mediating performance in exploratory tasks than in tasks involving unconditioned reflexive behavior. Thus, the interactions between TCAP and CRF in different brain regions may be one mechanism by which the differential behavioral responses are elicited in different paradigms.

Our results may also indicate that the interaction between TCAP and CRF is more complicated than a simple potentiation or blockade of effect, and may depend upon the baseline reactivity of the rats or the environmental context.

In conclusion, we have shown that 5 days of ICV treatment with TCAP-1 alters rats' behavioral response to an acute injection of CRF in the elevated plus maze, open field and acoustic startle tests. TCAP-1 treatment did not alter baseline behavior in these tests. However, when challenged with an acute CRF injection, rats previously treated with repeated TCAP injections showed a different response to CRF than did saline-treated controls. In the case of the locomotion and plus maze tests, TCAP-1 treated rats showed a potentiation of the CRF effect, whereas in the acoustic startle test, TCAP-1-treated rats showed an attenuation of the CRF effect. These data indicate that TCAP may interact with the CRF system in modulating anxiety-related behavior.

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