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Corticotropin-releasing factor (CRF)-induced behaviors are modulated by intravenous administration of teneurin C-terminal associated peptide-1 (TCAP-1)

Arij Al Chawaf[a], *Karen Xu*[c], *Laura Tan*[a,c], *Franco J. Vaccarino*[a,b,c], *David A. Lovejoy*[a], *Susan Rotzinger*[a,b,c,*]

[a] *Department of Cell and Systems Biology, University of Toronto, Toronto, Canada*
[b] *Department of Psychiatry, University of Toronto, Toronto, Canada*
[c] *Department of Psychology, University of Toronto, Toronto, Canada*

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ABSTRACT

The teneurin C-terminal associated peptides (TCAP) are a recently discovered family of bioactive peptides that can attenuate aspects of the behavioral stress responses of rats. Because TCAP has some structural similarity to the corticotropin-releasing factor (CRF) family of peptides, and modulates elements of the stress response, TCAP may act to modulate CRF actions *in vivo*. This hypothesis was tested by investigating anxiety-related behaviors in male rats following repeated intravenous (IV) TCAP-1 administration with either an acute intracerebroventricular (ICV) or IV CRF challenge. TCAP-1 alone did not affect behavioral responses significantly, however did significantly affect CRF-regulated behaviors depending on CRF's mode of injection. In both the elevated plus-maze and the open field tests, TCAP-1 had an anxiolytic effect on ICV CRF responses as indicated by decreased stretched-attend postures in the elevated plus maze ($p < 0.05$), and increased center time and center entries in the open field ($p < 0.05$). However, prior TCAP-1 treatment has an anxiogenic effect on the IV CRF-induced behaviors (decreased center entries and total distance in the open field ($p < 0.05$)). TCAP-1's actions are not mediated through acute changes in glucocorticoid levels and may occur via a central action in the brain. A fluorescently (FITC)-labeled TCAP-1 analog was IV-administered to investigate whether IV TCAP-1 has the potential to regulate central mechanisms by crossing the blood–brain barrier. FITC-TCAP-1 was detected in blood vessels and fibers in the brain indicating that uptake into the brain is a possible route for its interaction with CRF and its receptors. Thus, TCAP may modulate CRF-associated behaviors by a direct action in the CNS.

1. Introduction

The teneurin C-terminal associated peptides (TCAP) are a newly described family of bioactive peptides whose name reflects their close association with the teneurin transmembrane proteins [16]. The teneurin proteins were discovered in 1994 [3,15] as a novel transmembrane protein associated with *Drosophila* development. In vertebrates, the teneurins consist

* *Corresponding author at*: Department of Psychology, University of Toronto, 100 St. George Street, Room 4020, Toronto, ON, Canada M5S 3G3. Tel.: +1 416 978 5652; fax: +1 416 971 3190.
E-mail addresses: arij.alchawaf@utoronto.ca (A. Al Chawaf), karenxu@zoo.utoronto.ca (K. Xu), laura.tan@utoronto.ca (L. Tan), vaccar@psych.utoronto.ca (F.J. Vaccarino), dlovejoy@zoo.utoronto.ca (D.A. Lovejoy), rotzinger@psych.utoronto.ca (S. Rotzinger).
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of four highly conserved genes which play roles in signaling, neurite outgrowth and neural development [28].

The four versions of TCAP (1–4) are found in the terminal exon of the four vertebrate teneurins. The high degree of sequence similarity among all known TCAP orthologs and paralogs makes this family one of the most conserved peptide families known [16]. Synthetic versions of TCAP are active at modulating cAMP and protein levels, and regulating TCAP mRNA expression *in vitro* [22,30]. *In vivo*, acute administration of TCAP-1 modulates the acoustic startle response in rats and repeated administration of the peptide attenuated the acoustic startle response 3 weeks later [30]. We had theorized that TCAP-1 may induce long-term effects on neural circuitry and now have reported that synthetic mouse TCAP-1 regulates neurite outgrowth in immortalized hypothalamic neurons and primary cultures of embryonic mouse hippocampal cells [1].

In order to investigate the mechanism by which TCAP-1 modulates acoustic startle, we investigated the effects of the peptide on corticotropin releasing factor (CRF)-induced acoustic startle and found that intracerebroventricularly (ICV)-administered TCAP-1 could ablate the CRF-induced increase in acoustic startle [25]. Given that TCAP has some structural similarity with the CRF family of peptides, and that TCAP is also found outside the brain [16], we therefore examined the interaction of intravenously (IV)-administered TCAP-1 on IV and ICV-administered CRF-induced behaviors. In this study, we show that peripherally injected TCAP-1 significantly modulates CRF-regulated behaviors, but shows no modulation of the adrenal corticosterone response. Moreover, peripherally administered fluorescently-tagged TCAP-1 can be detected in the brain. Taken together, these studies suggest that TCAP-1's modulation of CRF-induced behavior may occur at a central level.

2. Materials and methods

2.1. Animals

Male Wistar rats ($N = 98$) weighing 250–300 g were obtained from Charles River Laboratories (Montreal, Canada). Rats were singly housed in Plexiglas shoebox cages (45 cm long × 20 cm wide × 25 cm high) under standard laboratory conditions (food and water available *ad libitum*, temperature 21 ± 1 °C, lights on at 07:00 h, off at 19:00 h). All testing took place between 09:00 h and 17:00 h. 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. Intravenous catheterization surgery procedure

For the experiments involving intravenous (IV) administration of TCAP-1 and CRF, the rats ($n = 40$) were implanted with IV catheters in the jugular vein. The rats were anaesthetized with isoflurane (4% induction, 2–3% maintenance in 100% O_2). IV catheters were constructed from Silastic® Laboratory tubing (12 cm, 0.51 mm I.D., 0.94 mm O.D., Dow Corning Corporation, Midland, MI) and irradiated PVC tubing (1 cm). The PVC tubing was placed outside of the Silastic tubing and fixed by soldering iron to divide the Silastic tubing into two ends of 8 cm and 3 cm long. The short end tubing was inserted into the right jugular vein and advanced into the heart. The position of the catheter was verified by withdrawing blood. The catheter was then flushed with heparinized sterile saline. The catheter was held in position using four non-absorbable sutures around the PVC tubing. The long end of the Silastic tubing was connected to a 23 G cannula (Plastics One, Roanoke, VA, USA), tunneled subcutaneously and externalized through a small incision on the head. The cannula was fixed to the skull with jewelers' screws and dental cement. The catheter was filled with heparinized saline (50 IU/ml, Heparin Leo, Belgium). The catheter was closed with a removable cap to prevent infection. The rats were kept warm under a lamp until regaining consciousness. To maintain catheter patency, the catheter was flushed with 0.1 ml of sterile heparinized saline daily.

2.3. Intracerebroventricular cannula implantation surgery

For the studies involving intracerebroventricular (ICV) administration of CRF and IV administration of TCAP, a separate group of rats ($n = 31$) were implanted with IV cannulae (according to procedure above) and ICV cannulae for the CRF administration. The rats were anaesthetized with 1–4% isoflurane/oxygen gaseous mix and fit into a rat 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 [21]. The cannula was fixed to the skull with jeweler's screws and dental acrylic. Dummy guides were placed into the cannulae to prevent blockage and to prevent debris from entering the brain. The rat was kept warm under a lamp until regaining consciousness.

2.4. Corticosterone assay following intravenous TCAP-1

A separate group of rats ($n = 19$) were prepared with IV catheters for corticosterone analysis. Following 1 week of recovery from the IV catheterization surgery, the rats were injected with one of four treatments: Vehicle (saline) ($n = 4$), CRF (300 pmol) ($n = 5$), TCAP-1 (300 pmol) ($n = 5$), or CRF + TCAP (300 pmol each) ($n = 5$) via the IV route. Thirty minutes after the injection, the rats were deeply anaesthetized with isoflurane, and blood (7–10 ml) was obtained into a sterile syringe by cardiac puncture using a 23 G needle. The blood was transferred to heparin-treated Vacutainer® tubes (Fisher Scientific, Canada), and centrifuged at 4000 rpm for 5 min. The plasma was transferred to 2 ml microfuge tubes and kept at −80 °C until time of analysis.

The plasma levels of corticosterone were measured by an ELISA kit according to the manufacturer's instructions (Assay Designs, Michigan, US). The sample was diluted 50 times with the assay buffer, and further diluted with steroid displacement reagent at a 2.5:97.5 ratio. Five corticosterone standards were prepared at final concentrations of 20,000, 4000, 800, 160, and 32 pg/ml. 100μl of each samples and standards were pipetted into the wells of the donkey anti-sheep IgG microtiter plate respectively, and 50 μl of corticosterone EIA antibody

was added to samples and standards. All samples and standard were run in duplicate.

After incubation at room temperature for 2 h, the plate was washed with 400 μl of wash solution three times, and 200 μl of the *p*-nitrophenyl phosphate solution in buffer was added to every well followed by 1 h incubation at room temperature. Then, 50 μl of stop solution was added to every well and the plate was read immediately on a spectrophotometer at 405 nm. The concentration of corticosterone using optical density was calculated using GraphPad software (GraphPad Software Inc., San Diego CA, 2003).

2.5. Drugs

Synthetic mouse TCAP-1 was synthesized and prepared for injection as previously described [30]. CRF (Sigma–Aldrich, Oakville ON) was dissolved in saline at a concentration of 1 μg/μl for ICV administration. Acute injections of CRF were administered 30 min prior to behavioral testing in the plus maze and open field tests. CRF was administered via either the ICV or IV routes as described. The ICV CRF injections were delivered in a volume of 1 μl at the speed of 2 μl/min by a syringe pump (Razel Scientific Instrument Inc., Stamford, USA). CRF was 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 cannula. CRF that was injected IV was prepared in a solution of 1 μg/105 μl saline, and administered in a volume of 150 μl using a 1 ml syringe.

2.6. Repeated TCAP-1 injections

Beginning 1 week after surgery, rats were injected once daily with 300 pmol (150 μl of 2×10^{-6} M) TCAP-1 or saline (for control) for 5 consecutive days via the IV route (see Fig. 1). The IV injection was delivered through the cannula fixed to the rat's skull and connected to the jugular vein, followed by an injection of heparinzed saline to flush the catheter. Following the five injection days, the rats were not injected or tested for a period of 6 days (see Fig. 1). On the 12th experimental day, the rats were tested in the elevated plus-maze, as described below. On the 16th experimental day, the rats were tested in the open field as described below.

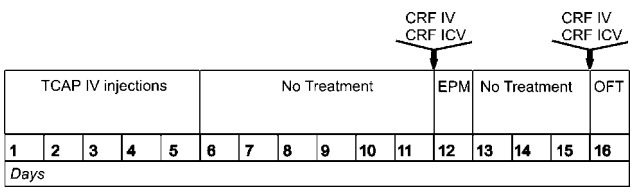

Fig. 1 – Timeline for the behavioral experiments. Experimental days are indicated horizontally across the bottom, treatment received on the corresponding days is indicated in the top row. An acute CRF challenge was injected 30 min prior to the behavioral tests and is indicated by the arrow (IV = intravenous, ICV = intracerebroventricular, EPM = elevated plus-maze, OFT = open field test).

2.7. Elevated plus-maze (EPM)

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) made of wood and painted black. The closed arms had walls made of black opaque plastic 50 cm high on three sides. The four arms were joined at the center by a 10 cm square platform. The apparatus was illuminated by a dim red light (25 W bulb).

On the 7th day after the end of the TCAP-1 injections, the rats were injected ICV with CRF (0 or 1 μg (210 pmol)) or IV with CRF (0 or 1.43 μg (300 pmol)). 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). The number of entries into, and time spent in open and closed arms were recorded. As well, the percentage of open arm time and entries was calculated [(open/total) × 100]. Video recordings were also analyzed by observers blind to treatments to quantify the occurrences of stretched-attend postures (exploratory movement in which the animal stretches forward without moving the hind legs and then retracts to the original position), head dipping (exploratory movement with the head extending over the edge of, and below the open arm) and rearing (exploratory movement in which the animal stands on its hind legs) [8,23].

2.8. Open field test

The rats were tested for locomotor activity in the open field on the 11th day after the end of the TCAP-1 injections (16th experimental day). The apparatus consisted of a 50 cm × 50 cm arena with 40 cm high walls made of black particle board. Thirty minutes prior to testing, the animals received injections of either saline or 210 pmol CRF (ICV), or saline or 300 pmol of CRF (IV). 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 and quantified 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.

Before the experiment, a 30 cm × 30 cm square in the center of the open field was defined as the center zone for data analysis. The total distance traveled in the entire chamber was calculated, as was distance traveled in the centre and border areas, the entries into the center area, and the time spent in the center and border areas.

2.9. Data analysis

Behavior in the plus maze and open field tests was quantified using the Ethovision® software. Statistical analysis of all data was carried out with GraphPad Prism® version 4.03. Data for each rat were converted to a percent of control, and the percent of control data was analyzed by one sample *t*-test

against a hypothetical mean of 100% (for control). Raw data for the corticosterone assay was analyzed by one-way analysis of variance with Tukey's post hoc tests. Data from rats with leaking or blocked catheters were excluded.

2.10. Preparation of the FITC-labeled peptide

Synthetic mouse TCAP-1 was synthesized as previously described [30] with a lysine at position 8, [K_8]-TCAP-1, replacing the naturally occurring arginine. The correct identity of the synthesized peptide was confirmed by a molecular mass determination using MALDI-TOF mass spectrometry on a sample of the purified material. The lyophilized peptide was solubilized in 50 mM borate buffer (pH 8.5) to a final concentration of 1 mg/ml. The [K_8]-TCAP-1 peptide was labeled with fluorescein isothiocyanate (FITC) according to the EZ-Label FITC Protein labeling kit (Pierce Biotechnology, Rockford, IL). FITC was dissolved in dimethylformamide, mixed with the [K_8]-TCAP-1 at a 24:1 excess molar ratio and incubated for 2 h at room temperature in the dark to allow for conjugation at the primary amine of the lysine group. A dextran column was used to remove the unconjugated FITC and the absorbance of each fraction was measured at 280 nm to determine the elution point of the conjugated peptide in phosphate buffered saline (PBS, 0.1 M phosphate, 0.15 M NaCl, pH 7.2). The four fractions with the highest protein absorbance readings were combined and sterilize-filtered using an Acrodisc Syringe 0.2 μm Supor low protein binding filter (Pall Life Sciences, Ann Arbor, MI). The filtrate was concentrated using a microsep 1K omega centrifugal device (Pall Life Sciences) at 4 °C. The samples were stored in the dark at 4 °C and injected within a day.

To confirm the size of the FITC-[K_8]-TCAP, 5 and 10 μl aliquots of the concentrated filtrate were run on a 10–20% Tris-Tricine gel. The amount of FITC required to be injected as a control was determined by applying different PBS-diluted preparations of FITC on a nitrocellulose membrane. The level of fluorescence was directly compared to that of a spot of the FITC-[K_8]-TCAP conjugate to be injected and that dilution used for the injection after sterilize filtration as above. Fluorescence was measured using an Epi Chemi II Darkroom system (UVP, Upland, CA) with the sequential integration function of LabWorks Image acquisition and analysis software (V4.0.0.8).

2.11. Intravenous injection of FITC-labeled TCAP-1

A separate group of rats ($n = 8$) was prepared with IV catheters as described above. Following 1 week of recovery, the animals were injected with 100 μl sterile 0.9% saline to flush the catheter and then injected with either 150 μl of saline ($n = 2$), FITC ($n = 2$) or FITC-[K_8]-TCAP ($n = 4$). All injections were given between 1000 and 1100 h. All animals then received 50 μl of saline to flush the catheter. The animals were returned to the colony for 1 h, after which they were deeply anaesthetized with isoflurane and decapitated. Each brain was collected in less than 1 min and snap-frozen in −70 °C isopentane.

2.12. Tissue processing of FITC-labeled TCAP brains

Each brain was mounted in the frontal plane on a cryostat and sliced into 40 μm-thick sections. Every fifth section was mounted on a gelatinized slide and overlaid with aqueous GelMount medium (Sigma–Aldrich Canada, Oakville, ON) and coverslips added. The slides were stored in darkness at 4 °C until visualized with fluorescence microscopy within 48 h. Digital image micrography of the brain slices was performed using a fluorescence Olympus (BX60) microscope fitted with a CCD CoolSNAP camera (Photometrics, Tuscon, AZ).

3. Results

3.1. Corticosterone assay

One-way analysis of variance revealed a significant effect of drug treatment on corticosterone concentrations ($F = 7.801$, $p = 0.0023$). Tukey's multiple comparison post hoc analysis revealed significant differences between Vehicle and CRF ($p < 0.01$), Vehicle and TCAP + CRF ($p < 0.05$), and between CRF and TCAP-1 ($p < 0.05$) (Fig. 2).

3.2. Elevated plus-maze (EPM)

A percent-of-control score was obtained for each of the TCAP-1-treated rats by dividing the rat's score by the mean of the corresponding control saline group and multiplying by 100. Percent-of-control data was analyzed by *t*-test against a hypothetical mean of 100% (for control). Data from rats that fell or jumped off the plus maze were excluded. Rats treated with TCAP + CRF(ICV) ($n = 7$) had a significantly lower number of stretched-attend postures (risk assessment) compared with rats treated with SAL + CRF(ICV) ($n = 7$, $t(6) = 3.093$, $p = 0.0213$) (Fig. 3A). The incidence of head dips and rearing did not differ between the groups.

The group treated with repeated TCAP + SAL(ICV) ($n = 5$) had significantly decreased rearing as compared with its control (SAL + SAL(ICV)) ($n = 4$) ($t(4) = 3.773$; $p = 0.0195$), but did not differ from control on any other measures (data not

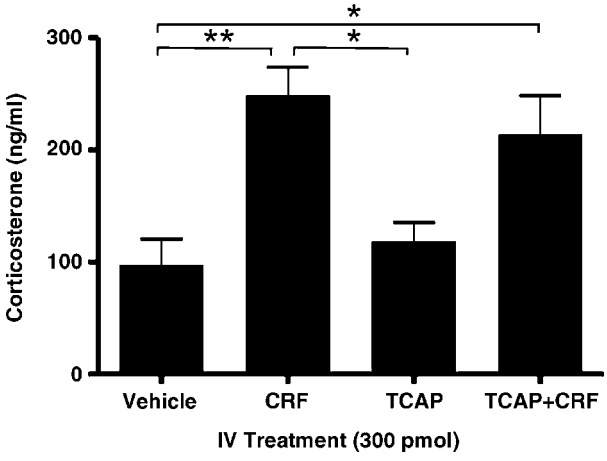

Fig. 2 – Corticosterone levels in rats treated with acute intravenous injection of Vehicle, TCAP-1, CRF, or TCAP + CRF (300 pmol each). Rats were sacrificed 30 min after injection and blood was collected via cardiac puncture. (*) Indicates significant difference at $p < 0.05$, (**) indicates significant difference at $p < 0.01$.

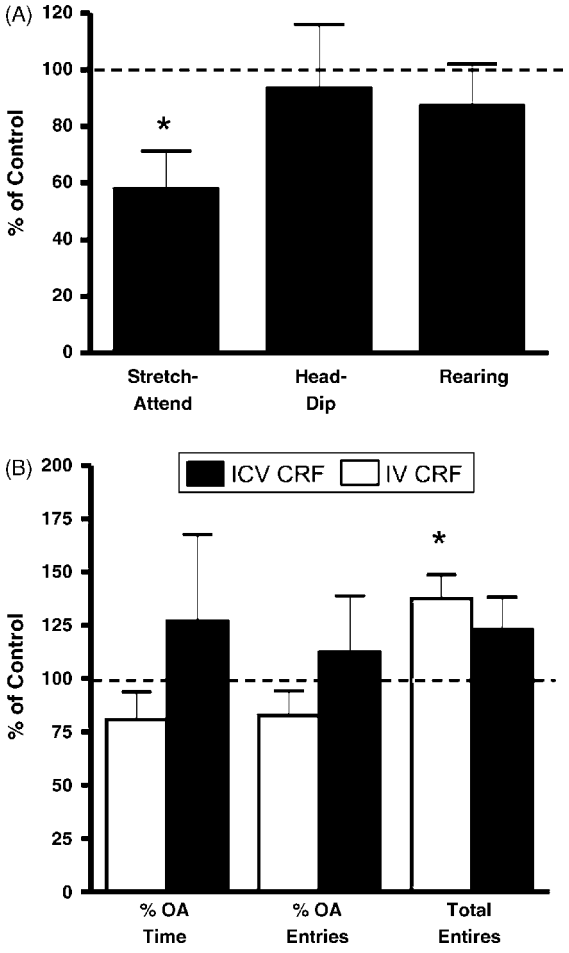

Fig. 3 – Elevated plus-maze behavior in rats treated previously with five IV TCAP-1 injections. Thirty minutes prior to testing, the rats were given an acute injection of CRF via either the ICV or IV route. Behaviors were coded for stretched-attend, head-dip and rearing behavior (A) as well as percent of time spent in open arms (%OA time), percent of open arm entries (%OA entries) as well as total entries into the arms (B). Results are presented as percent of control (TCAP-1-treated rats relative to saline-treated rats). Data were analyzed by *t*-test relative to a hypothetical mean of 100%. ((*) Indicates significantly different from 100%, $p < 0.05$).

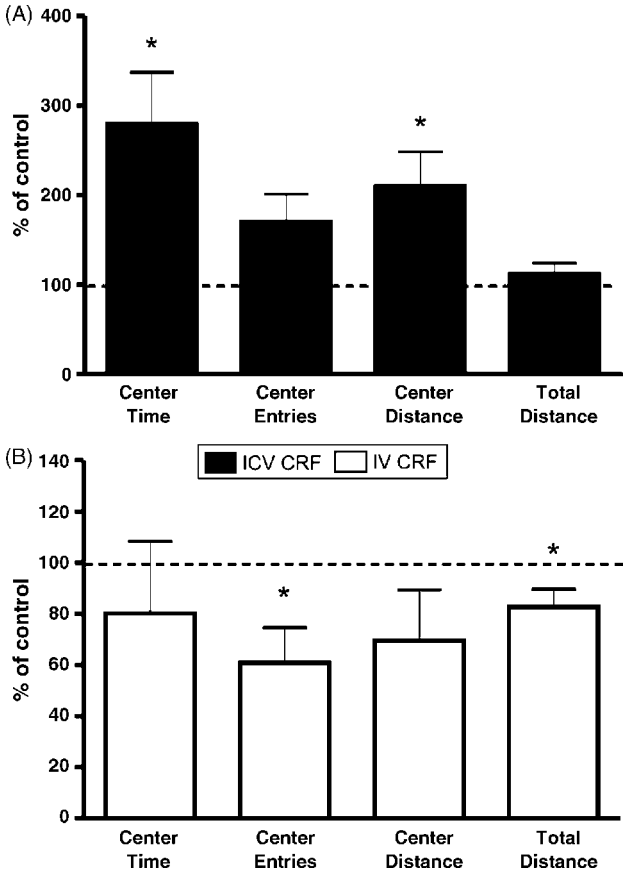

Fig. 4 – Open field behavior in rats treated previously with five IV TCAP-1 injections. Thirty minutes prior to testing, the rats were given an acute injection of CRF via either the ICV (A) or IV (B) route. Results are presented as percent of control (TCAP-1-treated rats relative to saline-treated rats). Data were tested by *t*-test relative to a hypothetical mean of 100%. ((*) Indicates significantly different from 100%, $p < 0.05$). Center time = time spent in central 30 cm × 30 cm of a 50 cm × 50 cm open field; center entries = total entries into center of field, center distance = distance (cm) traveled in center of field, total distance = distance traveled in the open field.

shown). There was a significant increase in total arm entries (percent of control) in the group treated with TCAP + CRF(IV) ($n = 7$), relative to the SAL + CRF(IV) group ($n = 6$, $t(6) = 3.339$, $p = 0.0156$) (Fig. 3B).

3.3. *Open field test*

Data from the first 10 min of the open field test were used in the analysis. Percent-of-control data was obtained as described above for the EPM. Center time ($t(6) = 3.123$, $p = 0.0205$) and center distance ($t(6) = 2.832$, $p = 0.0299$), were significantly increased in the group treated with TCAP + CRF(ICV) ($n = 7$) relative to control (SAL + CRF(ICV)) ($n = 7$), whereas border duration was significantly reduced ($t(6) = 3.120$, $p = 0.0206$) (Fig. 4A). There were no differences in total distance, center entries or border distance between the groups. The group treated with repeated TCAP + SAL(ICV) ($n = 7$) did not differ from control (SAL + SAL(ICV)) ($n = 6$) (data not shown).

For the rats treated with TCAP + CRF(IV) ($n = 7$) (Fig. 4B), there was a significant decrease in center entries ($t(7) = 2.848$, $p = 0.0248$) and total distance ($t(7) = 2.492$, $p = 0.0415$), relative to control (SAL + CRF(IV)) ($n = 6$). There were no differences on the other measures of locomotion. The group treated with repeated TCAP + SAL(IV) ($n = 4$) did not differ from control (SAL + SAL(IV)) ($n = 8$) (data not shown).

3.4. *FITC-[K_8]-TCAP uptake into the brain*

The results of the behavioral studies indicated that the IV-administered TCAP-1 may enter the brain directly. In order to

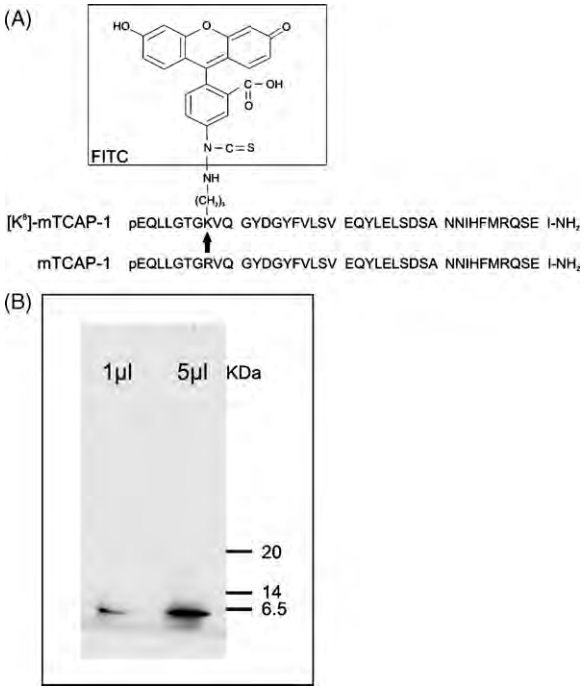

Fig. 5 – Model of labeled FITC-[K_8]-TCAP. (A) Synthetic mouse TCAP-1 with lysine at position 7 is attached to a FITC molecule. (B) Inverted-color image of gel with FITC-[K_8]-TCAP SDS-PAGE. Dark regions represent areas of fluorescence.

test this hypothesis, a fluorescent TCAP-1 peptide was synthesized and injected peripherally. Fluorescein isothiocyanate (FITC) was covalently linked to a TCAP-1 sequence with an amino acid containing a primary amine (lysine) at position 8. The resulting fluorescent peptide showed a molecular mass of 6 KDa as determined by SDS-PAGE (Fig. 5), which is in agreement to the theoretical mass of 4.9 KDa.

Peripheral injections of saline, FITC or FITC-[K_8]-TCAP were administered into the right jugular vein. After an hour, brains were removed, sectioned and scanned for fluorescence. The tissue sections from the brains of animals injected with either saline or FITC did not show any significant differences with respect to the presence and strength of fluorescence in the tissues. Numerous regions of the brain, for example, the pineal gland, primary olfactory cortex and the lateral lemniscus, showed varying levels of autofluorescence, observed as a diffuse region of brightness. However, these regions were evident in both the saline and FITC-injected animals.

In contrast, in the FITC-[K_8]-TCAP-injected rats, high levels of fluorescence were detected in several regions characteristically along large-sized blood vessels (20–40 μm diameter) and fibers. Most notable was the fluorescence in the caudate putamen region and fiber tracts of the alveolar hippocampus measuring 600–700 μm (Figs. 6 and 7); similarly high levels of fluorescence were observed in the anterior cingulate cortex, the cingulum and in tracts leading to the choroid plexus. Fluorescence along vessels and fiber tracts was not observed in control brains in those regions. Indeed, fluorescent capillaries were not observed in any of the FITC- or saline-injected rats.

4. Discussion

TCAP is a newly described bioactive neuropeptide that has been conserved across all species of vertebrates examined [16,22]. We have previously shown that TCAP administration into the basolateral amygdala or intracerebroventricularly alters the acoustic startle response [30]. These studies indicated that there may be lasting effects of TCAP administration on behavioral stress responses. CRF was delivered via the ICV or IV routes to help differentiate the effects of CRF acting on central extrahypothalamic CRF receptors from the effects of CRF-mediated HPA axis activation and glucocorticoid release, respectively. The present studies indicate that peripherally-administered TCAP-1 may cross the blood–brain barrier (BBB) and to modulate CRF-induced behaviors. Repeated TCAP-1 treatment by itself did not significantly affect behavior in the EPM or open field tests. However, rats that had previously been treated for 5 days with TCAP-1 had a *blunted* anxiogenic response to acute ICV CRF administration at 7–11 days after the TCAP-1 injections. Conversely, TCAP-1-treated rats that were treated acutely with IV CRF showed an *enhanced* anxiogenic response to CRF as compared with the saline-treated rats. Acute TCAP-1 injection did not directly activate the HPA axis, nor did it affect the activation of the HPA axis by CRF as evident by the corticosterone levels. Finally, evidence for direct uptake of TCAP-1 into the brain was examined using a synthetic fluorescently tagged version of TCAP-1.

In the EPM, rats given TCAP + CRF(ICV) were less anxious than rats treated with SAL + CRF(ICV), as indicated by a decrease in stretched-attend behaviors in the TCAP + CRF(ICV) group. Risk assessment measures (such as stretched-attend) may be more sensitive to anxiety-modulating drugs than traditional measures such as open arm time and entries [8,24]. Thus, although there were no differences in open arm time or entries, the decrease in stretched-attend postures is consistent with a decrease in anxiety. Use of ethological measures, and in particular stretched-attend postures, have proven valuable in identifying anxiolytic actions of drugs that conventional scoring methods may miss [24]. Stretched-attend postures are positively correlated with corticosterone levels in rats and mice [19,24] and inhibition of glucocorticoid synthesis inhibits stretched-attend postures while administration of corticosterone increases them [19]. No other parameters, such as open arm time or entries, or total entries, are affected by corticosterone inhibition or administration, nor is open field activity significantly affected [19]. Rodgers et al [24] also reported that corticosterone response is highly correlated with risk assessment, but not with open arm time or entries, general locomotor activity, rearing or head-dipping. Thus, our results are consistent with a decrease in anxiety in the TCAP + CRF(ICV) group, possibly via a glucocorticoid mechanism.

Rats treated with TCAP + CRF(IV) showed an increase in total arm entries in the EPM. Although this could be interpreted as an increase in general activity, there was no

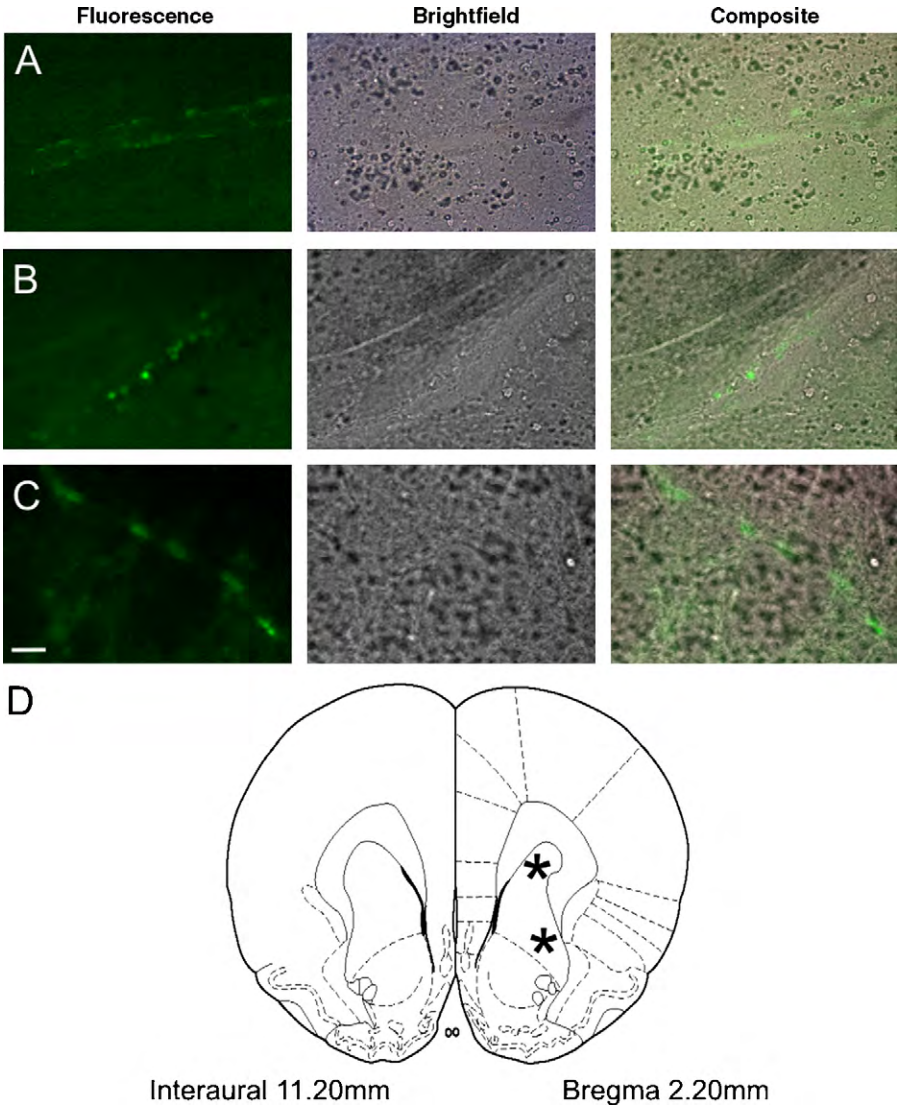

Fig. 6 – Representative coronal rat brain images of FITC-[K_8]-TCAP in fluorescence and brightfield mode, along with a composite. Fluorescence was observed along veins in the caudate putamen regions (A–C). (D) Schematic drawing of the brain slice [21], (*): regions of fluorescence, scale bar = 50 μm.

increase in activity in the open field in the TCAP + CRF(IV) group, as would be expected if it caused a general increase in activity. In fact, the TCAP + CRF(IV) rats had significantly lower total distance traveled in the open field than SAL + CRF(IV) (Fig. 4B). Total arm entries are influenced by the number of open and closed arm entries and, therefore, it is not a pure measure of activity, but can also reflect anxiety to a certain extent [23]. Rats treated with TCAP + CRF(ICV) showed an increase in center time and distance traveled in the center of the open field, as compared with rats treated with SAL + CRF(ICV). Again, this is consistent with an anxiolytic effect, or a blunting of the CRF effect. Conversely, when the acute CRF injection was delivered via the IV route, there were significant decreases in center entries and total distance traveled in the open field in the rats treated with TCAP + CRF(IV) as compared to the rats treated with SAL + CRF(IV). This behavioral profile is consistent with an increase in anxiety in the TCAP + CRF(IV) rats as compared with the TCAP + SAL(IV) rats.

In summary, IV injections of TCAP-1 resulted in different behaviors in response to IV or ICV CRF. Rats treated with repeated IV doses of TCAP-1, and then given an acute challenge injection of CRF (ICV) were less anxious than the rats that received repeated IV saline and CRF (ICV). This was indicated by decreased stretched-attend behaviors in the EPM and increased center time and distance in the open field in the TCAP-1-treated rats. Conversely, the rats treated with repeated IV TCAP-1, and a challenge injection of CRF via the IV route, were more anxious than their saline-treated counterparts, as indicated by decreased center entries in the open field test. The decrease in anxiety in the TCAP-1-treated rats given ICV CRF, as compared with the saline-treated controls, indicates that the TCAP-1 treatment may have affected CRF receptor function. The acute IV challenge of CRF is likely to activate only pituitary CRF receptors and, to activate the HPA axis, resulting in increases in circulating corticosterone. This would ultimately enter the brain and act

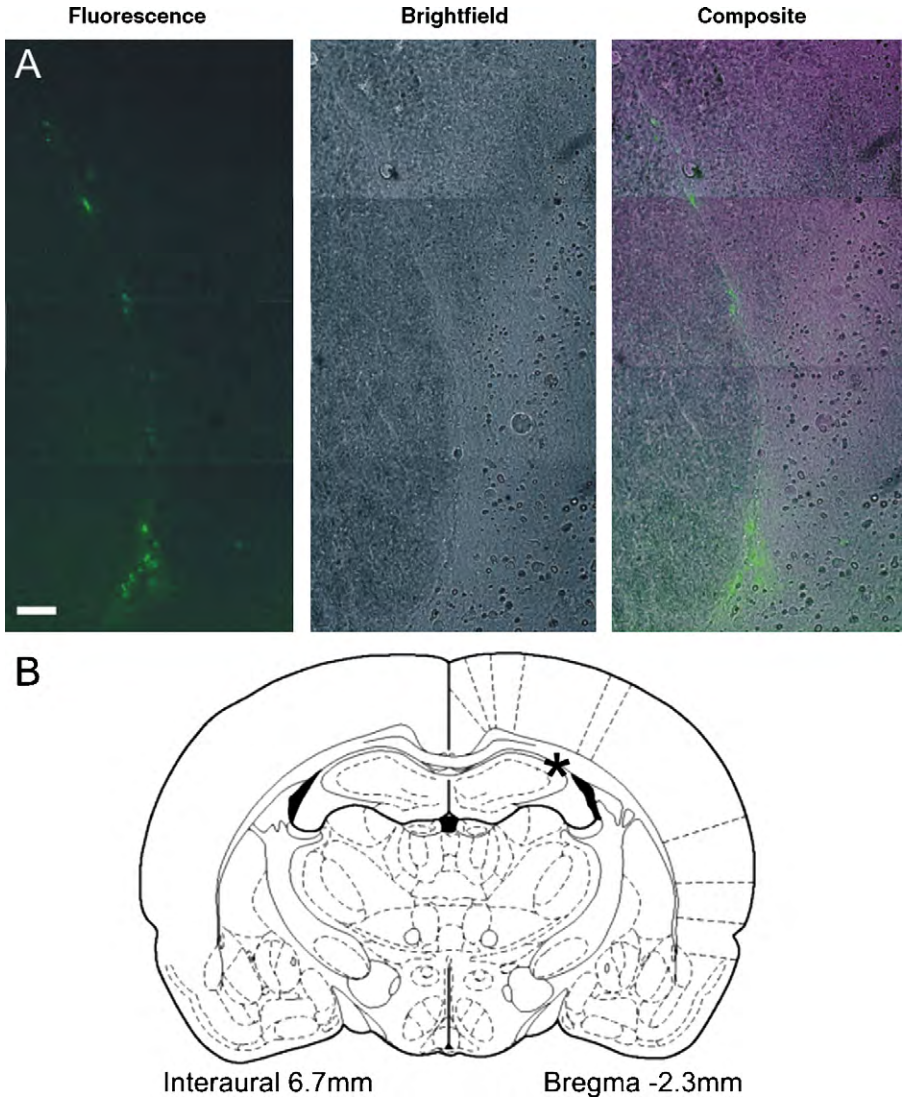

Fig. 7 – Representative coronal rat brain images of FITC-[K_8]-TCAP in fluorescence and brightfield mode, along with a composite. Fluorescence was observed along fibers in the alveolar hippocampus (A). (B) Schematic drawing of the brain slice [21], (*): region of fluorescence, scale bar = 50 μm.

on glucocorticoid GR receptors. However, CRF injected ICV can act on extrahypothalamic CRF receptors in stress-sensitive regions of the brain [6]. Thus, different behavioral effects are expected when CRF is administered via the IV and ICV routes. However, how TCAP-1 treatment differentially affects these outcomes is not yet known. It is possible that TCAP-1 administration affects glucocorticoid production and that this in turn affects CRF gene regulation [26]. However, acute administration of TCAP-1 did not affect the corticosterone response, although repeated administration might. Repeated TCAP-1 by itself might also affect CRF gene regulation, and future studies examining CRF mRNA after repeated TCAP-1 could address this question.

Finally, to investigate whether TCAP-1's observed effects are mediated centrally after crossing the BBB, we injected fluorescently-labeled TCAP (FITC-[K_8]-TCAP) to assess its penetration into the brain and subsequent distribution. This approach to track peptides with fluorescent labels in the brain has been used previously [5]. The use of a fluorescent label instead of the traditional radioisotope allows for a safer method of screening and for higher quality of *in situ* images. Previously, autoradiograms have been utilized to localize the region of uptake of radio-labeled peptides [2,17]. In our approach, we localized the peripherally-injected fluorescent TCAP-1 mostly in the capillaries of the caudate putamen as well as in fiber tracts in regions such as the hippocampus. Specific uptake into the regions of the hippocampus and the caudate nucleus was observed with thyrotropin-releasing hormone [34] and arginine–vasopressin [33].

Peptides cross the BBB by a number of mechanisms including fluid phase diffusion, adsorptive processes and receptor-mediated endocytosis [14]. A number of peptides that possess some structural similarity to TCAP [16], such as those belonging to the CRF and calcitonin families of peptides, have been reported to cross the BBB. Among the CRF-related paralogs, CRF has a saturable efflux system out of the brain

and a minor influx system into the brain, both of which are distinct from its urocortin paralog transport mechanisms. Urocortin gains entry into the brain after treatment with leptin and glucose. However, in cultured cerebral microvessel endothelial cells, more than 10% of fluorescently labeled urocortin can be exocytosed [20]. Urocortin 2 appears to enter the brain passively by diffusion whereas urocortin 3 is degraded by peptidases and does not enter the brain intact [4,13]. Many other peptides gain entrance to the brain [2,4,11]. Calcitonin gene related peptide (CGRP) and adrenomedullin, both paralogues of calcitonin, may also cross the BBB by diffusion via their vasoactive effects on the cerebral vasculature [7,9]. At present, the mechanism by which TCAP-1 may gain entry into the brain is unknown. Cognate receptors and protein transporter/binding mechanisms have yet to be elucidated. The peptide mRNA is found throughout the brain and is particularly highly expressed in the limbic region [30]. Immunoreactive forms of TCAP-1 are found throughout the brain and peripherally with particularly high expression in the testes (Al Chawaf, Chand and Lovejoy, unpublished observations) and appears to circulate in a bound form in blood (Trubiani, Al Chawaf and Lovejoy, unpublished observations). *In vitro*, TCAP-1 has a number of neuroprotective effects [27] similar to that described for adrenomedullin, CGRP [9,10,31] and vasoactive intestinal peptide [9]. TCAP-1 also appears to modulate the actions of CRF centrally [25,30].

Over the past 30 years, numerous peripherally injected peptides have been implicated with behavioral responses; however this does not necessarily imply passage across the BBB or even entry into the brain. For example, calcitonin induces feeding after central and peripheral administration but also specifically binds receptors in some circumventricular organs [29]. However, in our present study, we could not detect any fluorescence at any of the circumventricular organs. Any uptake of FITC alone is flushed from the central system within an hour, whereas FITC-[K_8]-TCAP is retained in other regions after entering the brain.

Other peripheral mechanisms have been used to explain the central and behavioral effects after peripheral administration of some peptides [18]. For example, the properties of the BBB may be altered by a vasoactive action of the peptides leading to an alteration of nutrient and ion intake into localized regions of the brain. Alternatively, secondary responses from receptors in the endothelium of brain capillary or at the ependymal lining of circumventricular organs may induce a central response. Another possibility is that peripherally injected peptides may have an action on some peripheral organs, such as the heart or kidney, which then affect the oxygen or osmoregulatory state of the brain which secondarily affects behavior. In our present study, however, we observed a number of fluorescence capillaries and fibers inside the brain suggesting that there was a direct uptake mechanism of the labeled peptide into the central nervous system.

In several locations, we observed fluorescence in small capillaries in the brains of the labeled [K_8]-TCAP-1 but not in the brains of animals treated with saline or saline and FITC. It has been argued that uptake across the BBB that surrounds the cerebral capillary network is statistically more likely simply in terms of the surface area. For example, the surface area of the choroid plexus [12] or circumventricular organs [13] is 3–4 orders of magnitude less than the total circumferential surface area of the brain capillaries. The endothelial lining of these capillaries appear to possess a number of aminopeptidases, carboxypeptidases and endopeptidases that have a wide range of specificities [32]. These enzyme systems could target TCAP-1 as well. Although these peptidases may limit the amount of peptide that crosses the BBB, some studies suggest that less than 0.1% of peripherally administered peptides may be necessary to trigger a central effect [13].

In summary, the present studies demonstrate that IV administration of TCAP-1 for 5 days has lasting (at least 11 days) effects on CRF-induced anxiety in the EPM and open field tests. Centrally-administered CRF resulted in a different behavioral profile in the TCAP-1-treated rats as compared with controls, and when administered centrally versus peripherally. This data is consistent with a central locus of action of TCAP-1. These studies are supported by the fluorescent uptake studies indicating that peripherally administered TCAP-1 gains entry to the brain.

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