

Multiple Behavioral Effects of Cocaine- and Amphetamine-Regulated Transcript (CART) Peptides in Mice: CART 42–89 and CART 49–89 Differ in Potency and Activity

ANTHONY W. BANNON, JENNIFER SEDA, MICHELLE CARMOUCHE, JONATHAN M. FRANCIS, MARK A. JAROSINSKI, and JAMES DOUGLASS

Amgen Inc., Neuroscience, Thousand Oaks, California (A.W.B., J.S., M.C., J.M.F., J.D.); and Amgen Boulder Inc., Peptide Chemistry, Longmont, Colorado (M.A.J.)

Received July 13, 2001; accepted August 23, 2001 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Cocaine- and amphetamine-regulated transcript (CART) encodes a neuropeptide precursor protein that is highly abundant in cells of the hypothalamus. To date, the major research focus into the function of CART peptides has been feeding behavior. However, CART mRNA is found in other areas of the brain as well as some peripheral tissues, suggesting possible broader functions of this peptide. In this study, we investigated the effects of two CART peptides, CART 42–89 and CART 49–89, in several behavioral assays. Peptides were administered by i.c.v. route of administration. Both CART 42–89 and CART 49–89 inhibited food intake with the minimally effective dose of CART 42–89 (0.5 μg) being 5-fold greater than that of CART

49–89 (0.1 μg). Both peptides also produced significant antinociceptive effects in the hot-plate assay with similar potency differences. CART 42–89 significantly inhibited the acoustic startle response (ASR) of pulse alone trials at doses of 0.1 and 0.5 μg . In contrast, CART 49–89 did not affect ASR of pulse alone trials at doses of 0.05 and 0.1 (μg). For prepulse inhibition (PPI) trials, in general, both peptides appeared to enhance the magnitude of PPI and CART 42–89 was less potent than CART 49–89. Overall, these data suggest CART peptides may have multiple roles in central nervous system function and there may be biological differences between two processed forms of CART peptide.

Cocaine- and amphetamine-regulated transcript (CART) is a putative neuropeptide precursor protein possibly involved in the regulation of ingestive behaviors (Kristensen et al., 1998). Two C-terminal CART-derived peptides, CART 42–89 and CART 49–89, have been isolated from rat hypothalamus and arcuate nucleus as well as the pituitary (Thim et al., 1999). Both peptides result from proteolytic cleavage events at dibasic residues (KR and KK, respectively) within the CART peptide precursor and thus represent potential biologically active neuropeptides. Intracerebroventricular (i.c.v.) administration of CART 42–89 decreases food intake in normal and in fasted rats (Kristensen et al., 1998; Vrang et al., 1999). After i.c.v. administration of CART peptide, there is an induction of c-fos mRNA expression levels in brain areas normally associated with control of feeding behavior and energy balance (Vrang et al., 1999, 2000). In addition, CART mRNA expression levels are modulated by different states of energy balance as well as leptin levels (Kristensen et al., 1998; Ahima et al., 1999). The high level of CART mRNA expressed in the hypothalamus (Douglass et al., 1995) also

supports a role for CART-derived peptides in the modulation of feeding behavior.

Although the hypothalamus is enriched with CART mRNA, *in situ* hybridization shows endogenous CART mRNA in other brain areas and peripheral tissues (Couceyro et al., 1997). Overall, most of these data indicate that CART peptide(s) is likely involved as a neuroendocrine modulator. However, as evidenced by the original discovery that showed up-regulation of CART mRNA in rat striatum after cocaine and amphetamine administration (Douglass et al., 1995), CART peptides may serve even broader functions. For example, there are published data indicating that CART peptide may produce an anxiogenic effect in rats (Kask et al., 2000) and that intrathecal administration of CART peptide in mice produces a hyperalgesic effect (Ohsawa et al., 2000). Based on the anatomical distribution of CART mRNA and peptides, it is speculated CART peptide may play a role in sensory processing as well as autonomic regulation (Couceyro et al., 1997; Koyle et al., 1998).

In the present investigation, the effects of two C-terminal

ABBREVIATIONS: CART, cocaine- and amphetamine-regulated transcript; ASR, acoustic startle response; PPI, prepulse inhibition; HPLC, high-performance liquid chromatography; RP-HPLC, reverse phase-high-performance liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; ANOVA, analysis of variance; pp4, prepulse level 4 dB above background; pp8, prepulse level 8 dB above background; pp16, prepulse level 16 dB above background.

CART-derived peptides, 42–89 and 49–89, were determined on several behavioral measures in mice, including those associated with sensory processing. These peptides were chosen because they appear to be the most abundant forms found in the hypothalamus and nucleus accumbens (Thim et al., 1999) and based on preliminary data in feeding studies demonstrating a potency difference (A.W. Bannon, unpublished observations). The behavioral assays used were food intake in fasted animals, open field locomotor activity, hot-plate, acoustic startle response (ASR), and prepulse inhibition (PPI) of acoustic startle.

Materials and Methods

Peptides

CART peptides were obtained by chemical synthesis at Amgen Boulder Inc (Longmont, CO) (Dodson et al., 1999). The sequence for rCART 42–89 is IPIYEKKGQVPM-CDAGEQCAVRKGARIGKLCDCPRGTSCNSFLLKCL and for rCART 49–89 the sequence is YGQVPMCDAGEQCAVRKGARIGKLCDCPRGTSCNSFLLKCL. Both peptides contain three disulfide bonds with C1/C3, C2/C5, C4/C6 connectivity (Thim et al., 1998; M. A. Jarosinski, W. S. Dodson, T. J. Zamborelli, B. J. Harding, D. M. Lenz, M. Haniu, and J. Douglass, unpublished observations).

Synthesis. Peptides were prepared at 0.1- or 0.2-mmol scale on an ABI 431 or 433 synthesizer (Applied Biosystems, Foster City, CA) by using Fmoc/OtBu protection strategy with both 1.0 mmol of *N,N'*-dicyclohexylcarbodiimide or *O*-benzotriazole-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate activation and Fmoc-Leu-Wang resin as the solid support. Removal of the α -amino Fmoc was with 20% piperidine in *N*-methylpyrrolidone.

Purification. Crude peptide and HPLC fractions were analyzed by analytical RP-HPLC with a Vydac C4 column (Vydac, Hesperia, CA) with a linear gradient of 20 to 40% acetonitrile (ACN) in 0.1% aqueous TFA over 25 min with a flow rate of 1 ml/min. Large-scale RP-HPLC was done with a Vydac 218TP C18 column (Vydac) by using a linear gradient of 10 to 70% ACN in 0.1% aqueous TFA over 60 min with a flow rate of 7 ml/min.

Mass Spectral Analysis. HPLC fractions were analyzed by flow injection with an API 1 electrospray-mass spectrometry (PerSeptive Biosystems, Framingham, MA). Liquid chromatography-mass spectrometry was done with an API 150 (PerSeptive Biosystems) in conjunction with Waters Integrity HPLC system (Waters Corporation, Milford, MA) by using YMC ODS-AQ C18 column, with a linear gradient of 5 to 55% ACN in 0.1% aqueous TFA over 10 min with a flow rate of 0.5 ml/min. Amino acid analysis was performed with ABI 420A derivatizer (Applied Biosystems) with a 130A separation system. rCART peptides were manually hydrolyzed in a vacuo by using 6 N HCl at 150°C for 60 min. RP-HPLC was performed on a Brownlee PTC column (Applied Biosystems) with a linear gradient of 2 to 11% ACN in water over 4 min, 11 to 27% over 6 min, and then 27 to 47% over 10 min.

Equilibrium Refolding. For refolding, 2.0 μ mol of fully reduced rCART peptides was dissolved in 10 ml of 50 mM Tris, 1 mM EDTA, and redox reagents 1 mM reduced glutathione/1 mM oxidized glutathione at pH 8.0. The reaction mixture was stirred at room temperature for 48 h then purified by preparative RP-HPLC, making sure the refold buffer was acidified. Disulfide mapping confirms connectivity (M. A. Jarosinski, W. S. Dodson, T. J. Zamborelli, B. J. Harding, D. M. Lenz, M. Haniu, and J. Douglass, unpublished observations) as previously reported (Thim et al., 1998).

Animals

Male 129svimj mice 8 to 12 weeks of age were obtained from the Jackson Laboratories (Bar Harbor, ME). This strain was chosen based on previous experience where stable behavioral performance

was found in the behavioral assays (e.g., prepulse inhibition). Animals were group housed (8–10/cage) and allowed at least a 7-day acclimation period to the American Association for the Accreditation of Laboratory Animal Care-approved animal facility at Amgen Inc. (Thousand Oaks, CA) before experiments. Mice were kept on a 12-h light/dark cycle with food and water available ad libitum at all times before the start of experiments. All experiments were conducted under protocols approved by Amgen's Institutional Animal Care and Use Committee and all studies were conducted during the light phase. Also, independent groups of animals were used for each experiment and for the testing of each peptide (i.e., animals were only used once).

Peptide Dosing

Mice were dosed using a free-hand i.c.v. (i.e., third ventricle) dosing technique. Briefly, mice were fully anesthetized (i.e., nonresponsive to tail pinch) using isoflurane. The injection site was determined by forming an equilateral triangle with the distance between the eyes as the base and the midline of the skull as the apex. A 25- μ l Hamilton syringe was fitted with a 27-gauge needle and a plastic guide was placed on the needle to allow only 3 mm to penetrate the skull. While holding the syringe perpendicular to skull, the mouse was injected at the apex with 5 μ l of solution and the needle was held in place for 10 s to prevent reflux. Mice were allowed 30 min of recovery before the start of testing in behavioral tests, unless otherwise noted. All peptides were diluted in sterile water and sterile water served as the vehicle (control). Control studies with dye were conducted to ensure the accuracy of injection. In these studies, the dye stayed confined to the ventricle system of the mouse (data not shown).

Open Field Locomotor Activity

Open field chambers (Hamilton-Kinder, San Diego, CA) were constructed of clear Plexiglas, with a base of 40.6 cm \times 40.6 cm, and walls at a height of 38.1 cm. Location and ambulation were measured by infrared photobeam breaks. A 16 \times 16 beam grid forms the first row at a height of approximately 3 cm from the floor. A second row of 16 photobeams in the x direction was located at a height of approximately 6.4 cm from the floor. The MotorMonitor software (Hamilton-Kinder) measures multiple endpoints, but for this study fine movements and total distance were used for statistical analysis. Fine movements were computed as all nonambulatory movements except rearing. Total distance (i.e., ambulation) was computed as distance traveled in the apparatus.

Fasting Studies

A single study was conducted with both peptides. The doses of CART 42–89 tested were 0.05, 0.1, and 0.5 μ g i.c.v. The doses of CART 49–89 tested were 0.01, 0.05, and 0.1 μ g i.c.v. These doses were based on data from preliminary studies indicating that at higher doses, there were gross motor disturbances that could disrupt the animals' ability to eat. Group-housed mice were deprived of food for approximately 20 h (i.e., overnight). The next day, animals were dosed with vehicle or peptide. After dosing, animals were placed in individual cages and a preweighted quantity of food was placed in the cage. Food intake measures were recorded 1, 2, 4, and 24 h after dosing.

Hot-Plate

A 30 cm \times 30 cm hot-plate analgesia meter (Columbus Instruments, Columbus, OH) set at 55°C was used for testing. The endpoint was the latency(ies) to front paw lick, or until 25 s had elapsed, after which the animals were immediately removed from the plate. Testing began 45 min after treatment with peptides.

2, and 4 h after treatment with decreases of 80% ($p = 0.0002$), 60% ($p < 0.0001$), and 43% ($p = 0.02$), respectively. Overall, only the high doses of these CART peptides significantly inhibited food intake and the minimally effective dose of CART 42–89 (0.5 μg i.c.v.) was 5-fold greater than that of CART 49–89 (0.1 μg i.c.v.).

Effects of CART Peptides on Thermal Nociception (Hot-Plate). Hot-plate data for CART 42–89 and CART 49–89 are presented in Fig. 2. With CART 42–89 there was an overall effect of dose [$F(3,28) = 10.363$, $p < 0.0001$]. Post hoc analysis indicated a significant increase in latency to respond at 0.5 μg (i.c.v.) 42–89 ($p < 0.0001$). An overall significant effect of dose was found with CART 49–89 on paw lick latency [$F(3,27) = 3.476$, $p = 0.03$]. Post hoc analysis indicated a significant increase in paw lick latency with 0.1 μg (i.c.v.) CART 49–89. Based on these data, the dose of CART 42–89 that significantly increased paw lick latency (0.5 μg i.c.v.) was 5-fold greater than that of CART 49–89 (0.1 μg i.c.v.).

Acoustic Startle Response. Built into a paradigm of prepulse inhibition of acoustic startle were pulse alone trials (120 dB) (under *Materials and Methods*). These pulse alone trials were used to compute the ASR. With CART 42–89 (Fig. 3A), there was an overall effect of dose [$F(2,45) = 8.941$, $p = 0.005$] on ASR. Post hoc analysis indicated that 0.1 and 0.5 μg i.c.v. CART 42–89 significantly decreased ASR by 37% ($p = 0.005$) and 52% ($p = 0.002$), respectively. Interestingly, with CART 49–89 (Fig. 3B), no effect on ASR was observed [$F(2,44) = 0.217$, $p = 0.8$] with the doses tested (0.05 and 0.1 μg i.c.v.).

Prepulse Inhibition. In the PPI paradigm, three different prepulse levels were used: 1) 4 dB above background (pp4), 2) 8 dB above background (pp8), and 3) 16 dB above background (pp16). In a separate experiment with CART 42–89 (Fig. 4A) and CART 49–89 (Fig. 4B), prepulse level-dependent inhibition was observed in vehicle-treated animals in both experiments. For CART 42–89, there was not a significant overall effect of treatment [$F(2,45) = 2.386$, $p = 0.1$] but there was a significant effect of the within-subjects measure of prepulse level [$F(2,90) = 241.409$, $p < 0.0001$] and a significant interaction of prepulse level and treatment [$F(4,90) = 3.928$, $p = 0.006$]. At the pp8 level,

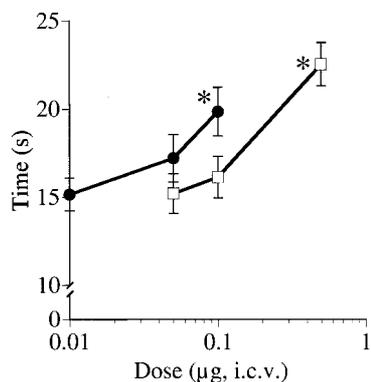


Fig. 2. Mean \pm S.E.M. paw lick latency(ies) in the hot-plate assay after i.c.v. dosing with CART peptides. Separate experiments were conducted for each peptide. The mean \pm S.E.M. latency(ies) for the vehicle group in the experiment with CART 42–89 was 14.5 ± 1.1 . The mean \pm S.E.M. latency(ies) for the vehicle group in the experiment with CART 49–89 was 15.5 ± 0.8 . *, significantly different from respective vehicle-treated group, $p < 0.0001$. $n = 7$ –8/group. □, CART 42–89; ●, CART 49–89.

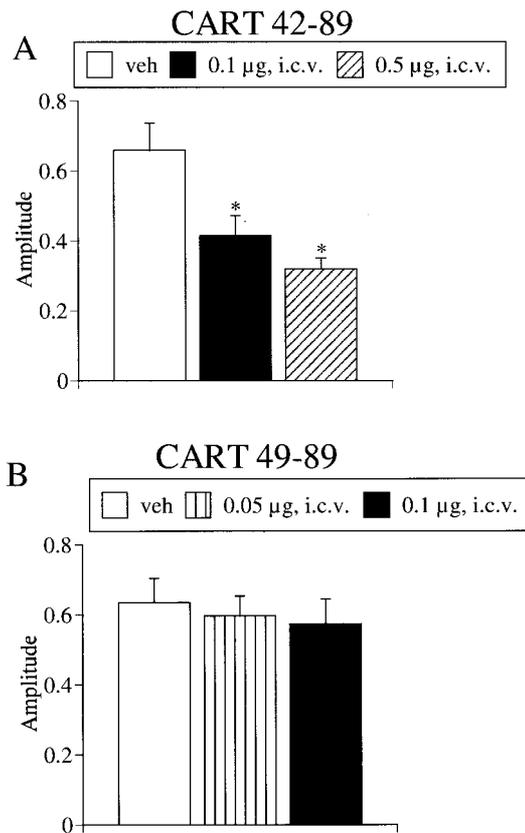


Fig. 3. Mean \pm S.E.M. amplitude of startle response to a 120-dB acoustic stimuli after i.c.v. dosing with CART 42–89 (A) and CART 49–89 (B). *, significantly different from respective vehicle-treated group, $p < 0.006$. $n = 15$ –16/group.

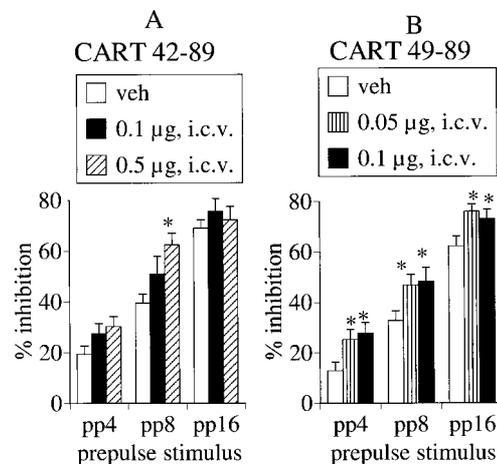


Fig. 4. Mean \pm S.E.M. percentage of inhibition of acoustic startle response at pp4, pp8, and pp16 above background after i.c.v. dosing with CART peptides. *, significantly different from respective vehicle-treated group at same prepulse level, $p < 0.05$. $n = 15$ –16/group.

CART 42–89 at the 0.5 μg i.c.v. dose produced a significant 58% increase ($p = 0.004$). With CART 49–89, an overall significant effect of treatment [$F(2,44) = 4.880$, $p = 0.0122$] and a significant effect of the within-subjects measure of prepulse level [$F(2,88) = 323.803$, $p < 0.0001$] but no interaction [$F(4,88) = 0.466$, $p = 0.76$] was found. At pp4, post hoc analysis indicated significant increases in PPI of 96% ($p = 0.03$) and 115% ($p = 0.01$) with 0.05 and 0.1 μg i.c.v. CART 49–89, respectively. At pp8, post hoc

analysis indicated significant increases in PPI of 43% ($p = 0.03$) and 47% ($p = 0.02$) with 0.05 and 0.1 μg i.c.v. CART 49–89, respectively. At pp16, post hoc analysis indicated significant increases in PPI of 22% ($p = 0.01$) and 17% ($p = 0.04$) with 0.05 and 0.1 μg i.c.v. CART 49–89, respectively.

Discussion

Most of the published *in vivo* work with CART peptides has reported on CART 42–89 with the focus on feeding behavior (Kristensen et al., 1998; Vrang et al., 1999). Data from the current study suggest that CART peptides have a diverse spectrum of biological activity and two biologically relevant forms of CART peptide can be distinguished *in vivo* based on potency or activity (i.e., ASR). There is one published study that compares the effects of i.c.v. administration of CART peptides on food intake in fasted mice with peptides corresponding to CART 42–89 and CART 49–89 (Thim et al., 1998). In that study, CART 42–89 appears to be more potent and efficacious than CART 49–89. Those results are in contrast to the current study in which CART 42–89 appears to be 5-fold less potent than CART 49–89 with respect to effects on food intake. However, comparison of the two studies is difficult because of differences in the experimental protocols (e.g., method of CART peptide production and strain of animals used). Overall, the data from the present study provide evidence across multiple models that the two forms of CART peptides tested can be differentiated.

In the first published report describing the effects of CART peptide injection (CART 42–89) in rats there was a note of “motor effects” at doses that affected food intake, but additional data were presented suggesting the motor effect was not underlying the effect on food intake (Kristensen et al., 1998). We have previously monitored home-cage activity concurrently with feeding (A.W. Bannon, unpublished observations). In those studies, there was a trend for CART peptides to decrease locomotor activity at doses that inhibited food intake but the duration of the effect on food intake was longer (4 h) than that on locomotor activity (1 h). In the current study, CART peptide treatment had no statistically significant effects on parameters taken from a 1-h session in the open field locomotor assay. The lack of effect on motor activity in this experiment could be due to the timing. For example, it is possible that motor effects occurred immediately after injection and this effect would not have been found because testing began 30 min after injection. Also, there was a high degree of variability that could underlie the lack of observing an effect. However, the important point in the current study was that gross motor impairment was not observed using doses of CART peptides examined in subsequent behavioral assays. This was an important finding because all of the behavioral assays used in this study depend upon a motor response.

In this study, only the highest dose of CART 42–89 (0.5 μg i.c.v.) and CART 49–89 (0.1 μg i.c.v.) decreased food intake with no evidence of dose dependence. As noted above, gross motor impairment was not observed at these doses, but these results may bring into question the specificity of the anorectic effects of these peptides. A recent article by Aja et al. (2001) that used some microstructure analysis suggests altered oral motor function and/or changes in perception of

palatability could contribute to the anorectic action of CART peptide (e.g., 42–89) in rats. As suggested by those authors, further microstructure analysis of feeding may help clarify the nature of the anorectic effect of CART peptides.

One novel finding in the current study was that CART peptides produced a significant antinociceptive effect in the hot-plate assay and there was a potency difference between the two peptides. The antinociceptive effect in the present study after i.c.v. dosing of CART peptides was in contrast to a hyperalgesic effect observed after intrathecal dosing of CART peptide corresponding to CART 42–89 (Ohsawa et al., 2000). However, differences in route of administration could contribute to the differences observed behaviorally. The mechanism(s) for CART peptides modulating nociception is unclear, but CART peptide positive immunoreactivity has been found in areas associated with the modulation of nociception such as the periaqueductal gray, the dorsal raphe nucleus, the locus ceruleus, and the spinal cord (Douglass et al., 1995; Koylu et al., 1998). Thus, CART peptides may modulate release of neurotransmitters involved with pain processing and/or transmission. Further study into the involvement of CART peptides in nociception is needed to confirm these initial findings. Regardless, as observed with feeding, in the hot-plate model CART 42–89 was 5-fold less potent than CART 49–89.

Another novel finding in this study was that CART 42–89 (0.1 and 0.5 μg i.c.v.) decreased ASR but at the doses tested, CART 49–89 (0.05 and 0.1 μg i.c.v.) had no effect on ASR. This was an interesting finding because it was the only example of CART 42–89 having an effect that was not observed with CART 49–89, at least at the doses tested. ASR is a simple reflexive response that is subject to different forms of plasticity. For example, there have been demonstrations that secondary reinforcers may diminish the ASR (Koch et al., 1996) and aversive stimuli can increase ASR (Davis et al., 1993). CART was originally discovered as an up-regulated mRNA in striatum after acute treatment with cocaine and amphetamine, and there has been previous speculation about its potential role in underlying the addictive properties of these stimulants (Douglass et al., 1995; Hurd et al., 1999). In mice, amphetamine administration has been shown to decrease ASR (Dulawa and Geyer, 1996; Ralph et al., 1999). One interpretation for the current finding of CART 42–89 decreasing ASR was that this peptide produced a reinforcing effect. This interpretation is consistent with a finding by Kimmel et al. (2000) in that in rats intraventricular injection of CART peptide (e.g., 42–89) produced significant place preference.

Interestingly, treatment with CART 42–89 tended to enhance PPI, and a similar but more robust effect was observed with CART 49–89. Thus, despite different effects on ASR, these peptides both appear to enhance PPI. PPI is a measure of sensorimotor gating. Statistically, only at the prepulse level of 4 dB above background (pp4) was the effect of CART 42–89 (0.5 μg i.c.v.) significant and this dose was 10-fold greater than the dose of CART 49–89 (0.05 μg i.c.v.) observed to enhance PPI at the same prepulse level. This difference in potency between the peptides was consistent with the effects observed on feeding and in the hot-plate assay. The finding that CART peptides modulated PPI was not too surprising. This was because the brain areas thought to mediate PPI such as the ventral tegmental area, nucleus accumbens, and

ventral pallidum (Nauta et al., 1978; Swerdlow et al., 1990, 1992; Swerdlow and Geyer, 1993; Kodosi and Swerdlow, 1995) are positive for CART mRNA and peptide immunoreactivity (Douglass et al., 1995; Couceyro et al., 1997; Koyle et al., 1998). However, the apparent enhancement of PPI could be considered surprising. If anything, one may have predicted that increasing brain CART peptide levels would possibly disrupt PPI. This was based on the findings that amphetamine, an agent that increases CART mRNA levels in the striatum, also disrupts PPI in rats and mice (Mansbach et al., 1988; Dulawa and Geyer, 1996). Disruption of PPI has been found in certain psychiatric disorders such as schizophrenia. It was tempting to speculate that enhancement of PPI in normal animals by CART peptide treatment represented a potential novel target for disorders such as schizophrenia. However, it should be noted that not all known effective antipsychotic drugs enhance PPI in normal animals and some drugs that are not effective in schizophrenia do enhance PPI in normal animals (Depoortere et al., 1997). Thus, there seems to be little predictive value of enhancement of PPI in normal animals for identifying potential schizophrenia treatments. It will be interesting to further study the effects of CART peptides in models relevant for neuropsychiatric diseases.

In summary, this work demonstrates multiple behavioral actions of CART peptides in mice after i.c.v. administration and that two biologically relevant forms of CART can be differentiated based on minimally effective dose in several assays as well as activity (i.e., effect on acoustic startle response). Recently, NMR spectroscopy has determined the three-dimensional structure of the disulfide-linked C-terminal part of human CART molecules corresponding to rCART 42–89 and rCART 49–89 (Ludvigsen et al., 2001). The specific disulfide-binding pattern between the six cysteine residues determines the major structural component of the molecule, a compact cystine knot. In contrast, the N-terminal residues have a comparatively disordered structure. Thus, these CART peptides are predicted to have a “ball and chain” structure (Thim et al., 1998), with the length of the N-terminal chain portion possibly serving to structurally distinguish the two CART peptides tested here. The fact that these two CART peptides can now be differentiated both structurally and functionally is suggestive of multiple CART receptors. Obviously, identification of mammalian CART receptors will be critical in further elucidating the function of these putative neuropeptides. The results from the current study suggest that CART 42–89 and CART 49–89 may be useful tools for identifying CART receptors, and that CART is a potentially meaningful target for the modulation of multiple central nervous system-mediated behaviors.

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Address correspondence to: Dr. Anthony Bannon, Amgen Inc., One Amgen Center Dr., M/S 29-1-A, Thousand Oaks, CA 91320-1789. E-mail: abannon@amgen.com