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Cocaine- and amphetamine-regulated transcript (CART) peptide activates the extracellular signal-regulated kinase (ERK) pathway in AtT20 cells via putative G-protein coupled receptors

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Abstract

CART peptides are important neurotransmitters, but little is known about their receptors or signaling pathways in cells. In this study we describe the effects of CART 55–102 on the stimulation of extracellular signal-related kinase (ERK) in a pituitary-derived cell line. CART 55–102 treatment resulted in markedly enhanced ERK phosphorylation in AtT20 and GH3 cells, but had no significant effect on ERK phosphorylation levels in a variety of other cell types that were examined. The peptide activated ERK1 and 2 in AtT20 cells in a dose- and time-dependent manner, but an inactive peptide, CART 1–27, had no effect. U0126, an inhibitor of the MEK kinases, blocked the CART-stimulated activation of ERKs. ERK activation was also attenuated by pertussis toxin pre-treatment, but not by genistein, suggesting a Gi/o-dependent mechanism. Overall, these data strongly support the existence of a specific receptor for CART peptide that is a G-protein coupled receptor utilizing a Gi/o mechanism involving MEK1 and 2.
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Keywords: CART; CART peptide; CART receptor; ERK activation

CART peptides (CART 55–102 and CART 62–102) are neurotransmitters and endocrine factors in the nervous system and periphery [8]. They have been implicated in the regulation of feeding and body weight, drug reward and reinforcement and other processes [8]. They also have neuroprotective properties and promote the survival and differentiation of neurons in vitro [12]. CART peptides appear early in the development of the CNS and other tissues suggesting a role in the development of specific regions of the brain [4], GI tract [6], pancreas [16] and ovary [11]. While the importance of CART peptides is clear, little is known about the cellular mechanisms by which CART peptides exert their effects. No receptor for CART peptide has yet been identified, but some cellular effects have been observed, notably modulation of voltage-gated calcium signaling [17]. Also, injection of CART 55–102 into the lateral ventricles has been found to cause an increase in c-Fos levels in a variety of neurons [14]. In an effort to elucidate further the cellular signaling effects of CART peptides, and because of the observed effects of CART peptide on differentiation and development [12], we have examined the ability of CART 55–102 to alter the MAPK cascade and the phosphorylation state of ERK1 and 2. ERKs are known to be activated by a variety of growth factors, hormones and neurotransmitters, and we have found that CART peptide stimulation strongly regulates ERK activity in pituitary-derived cell lines.

AtT20 cells, a mouse pituitary cell line, were maintained in DMEM medium supplemented with 10% horse serum at

Abbreviations: CART, cocaine- and amphetamine-regulated transcript; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; MEK, MAPK kinase; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinases; G, genistein; PTX, pertussis toxin

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37 °C under an atmosphere of 5% CO_2 in air. 1×10^5 cells were seeded to 20 mm dishes. After starving, the cells for 12–16 h in DMEM medium supplemented with 0.5% horse serum, the cells were pre-treated with 150 μM genistein (Sigma Chemical, St. Louis, MO) for 20 min or 200 ng/ml pertussis toxin (PTX) (Sigma Chemical, St. Louis, MO) for 16 h or 10 μM U0126 (Cell Signaling Technology, Inc., Beverly, MA) for 30 min. Subsequent to pre-treatment with inhibitors, the medium was supplemented with 1 nM, 10 nM, 100 nM, 1 μM or 10 μM CART 55–102 for 0, 2, 4, 5, 10, 15, 20 or 30 min in the presence or the absence of inhibitors or with 5% fetal bovine serum (FBS) serving as a positive control for the experiment. PC12 cells, a rat pheochromocytoma cell line, and GH3 cells, a rat pituitary cell line, were maintained in Ham's F12K medium supplemented with 15% horse serum and 2.5% FBS. CATH.a cells, a mouse locus coeruleus cell line, were maintained in RPMI 1640 medium supplemented with 8% horse serum and 4% FBS. HEK239 cells, a human embryonic kidney cell line, were maintained in DMEM medium supplemented with 10% FBS. Neuro-2a (N2a) cells, a mouse neuroblastoma cell line, were maintained in EMEM-modified medium supplemented with 10% FBS. All the cell lines were handled and treated similarly to AtT20 cell line. The cell lines, the media, and the sera were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Total protein was extracted in 50 μl lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 nM okadaic acid, 1 μg/ml leupeptin and 1 mM PMSF. 15–20 μg total protein was loaded in 1× sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol and 0.01% (w/v) bromphenol blue) onto 4–20% Novex® precast SDS-Tris-Glycine gel (Invitrogen, Carlsbad, CA). After overnight protein transfer at 30 V, the membranes were blocked with 5% non-fat milk in 1× TBS-T (Tris-Buffered Saline, 0.1% Tween-20 (pH 7.6)) for 1 h, and then incubated with the P-ERK1/2 (1:1000) antibody overnight. Bacterially expressed, fully phosphorylated ERK2 protein served as a positive control for Western blotting experiments. All antibodies and control protein were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and the manufacturer's instructions were followed. The signal was detected by using horseradish peroxidase (HRP)-conjugated anti-rabbit (1:1000) antibodies, and an enhanced chemiluminescence kit (ECL; Amersham, Arlington Heights, IL). Membranes were stripped with Restore™ Western Blot Stripping (Pierce, Rockford, IL) and reprobed with ERK1/2 antibody (1:1000) in order to standardize the results and provide a positive control for Western blotting experiment. The multiple images of Western blots were acquired using camera-based image acquisition (Instant Camera System, Poland). In accordance with the Scion Image instruction, the gray scale (8-bit; 1–254 gray levels) TIFF file image analysis was carried out using Scion Image Software (NIH, Bethesda, MD, USA).

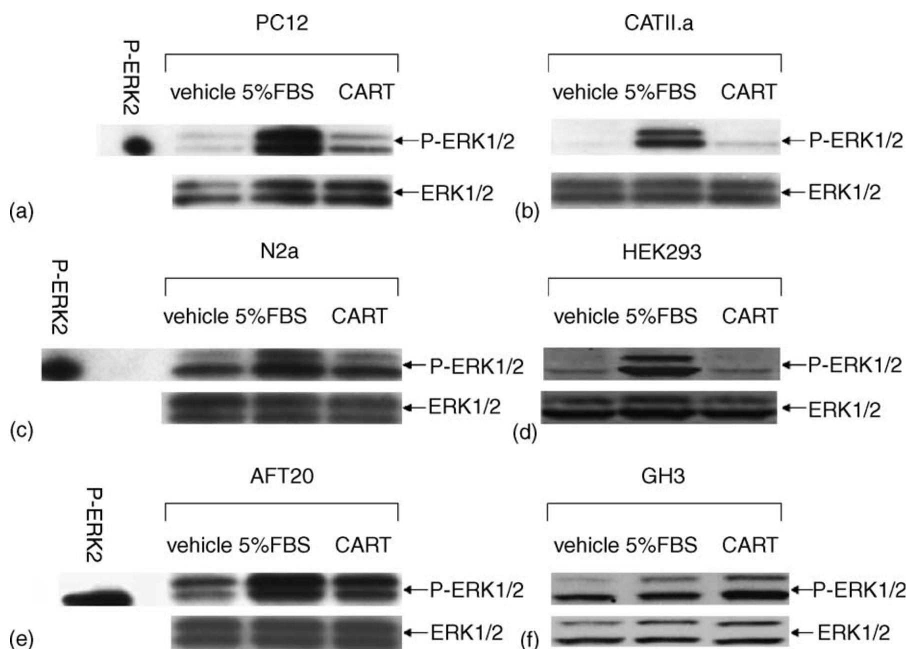

Fig. 1. Activation of ERK in PC12, CATH.a, N2a, HEK293, GH3 and AtT20 cells is demonstrated by Western blotting. All six-cell types were stimulated with either vehicle, 1 μM CART 55–102 or 5% fetal bovine serum (FBS) for 4 min. All cells showed an increase in ERK phosphorylation in response to FBS, but only GH3 and AtT20 cells exhibited enhanced phosphoERK (P-ERK) immunoreactivity in response to CART stimulation ($n = 3$ or more for each cell type). Each panel shows the portion of the Western blot containing P-ERK1 and 2 following treatment (upper), as well as the blot stripped and reprobed for ERK1 and 2 (lower). Distinct P-ERK1 and 2 bands are shown in the upper blot and distinct ERK1 and 2 bands are shown in the lower blot. The P-ERK2 standard is shown on the extreme left verifying the location of P-ERK1 and 2 in the upper blot.

Briefly, the pixel values were obtained by outlining the bands and a similar area adjacent to but separated from the bands was measured for background values which were subtracted. Then the values of phosphorylated bands were divided by values of non-phosphorylated bands to provide relative values shown in bar graphs.

GraphPad Prism was utilized for data analysis. Data are presented as mean ± S.E.M. of at least three independent experiments. Statistical analyses were carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and $p < 0.05$ was considered statistically significant. Bar graphs were used to show averages of several experiments.

A variety of cell types were examined for increased levels of phosphorylated ERKs (P-ERKs) in response to application of CART 55–102 peptide. HEK293, PC12, N2a and CATH.a cells gave little or no response, but pituitary-derived AtT20 and GH3 cells gave consistent and robust responses (Fig. 1). AtT20 cells yielded the largest CART-induced effects on ERK phosphorylation state (normalized data) and therefore were used in all subsequent experiments. CART 55–102 (100 nM) was incubated with AtT20 cells for varying times, and levels of P-ERK were determined by Western blotting (Fig. 2). Levels of P-ERK were significantly different over time (one-way ANOVA, $F_{(5,23)} = 13.36$, $p < 0.0001$). P-ERK was increased at 2 and 5 min, but was back to control levels at 10, 15 and 20 min. The result of Tukey's post hoc tests showed that relative P-ERK levels at 5 min were significantly different ($p < 0.001$) compared to those at 0, 10, 15, and 20 min; 2 min treatment was significantly different ($p < 0.01$) compared to 15 min treatment. A dose–response curve for the effects of CART peptides on ERK phosphorylation is shown in Fig. 3. Levels of P-ERKs increased with 10 nM, 100 nM and 1 μM concentrations of CART 55–102 peptide and peaked at 10 μM CART 55–102 peptide, which was the highest dose tested. The inactive CART (iCART) peptide, CART 1–27, however, had no effect on ERK phosphorylation state (Fig. 3). A one-way ANOVA showed significant differences $F_{(6,27)} = 12.22$, $p < 0.0001$. Tukey's test was used for post hoc analysis. Values at 10 μM were significantly different ($p < 0.001$) compared to vehicle, iCART and 1 nM. Values at 10 μM were also different from 10 nM ($p < 0.01$). Values at 1 μM CART were significantly different ($p < 0.01$) compared to vehicle, iCART. Values at 1 μM were also different from 1 nM ($p < 0.001$). Values at 100 nM were different from those at 1 nM ($p < 0.05$).

Having established that CART 55–102 peptide can activate the ERKs in these cells, we next proceeded to elucidate the mechanism. Both G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) can activate ERKs [3].

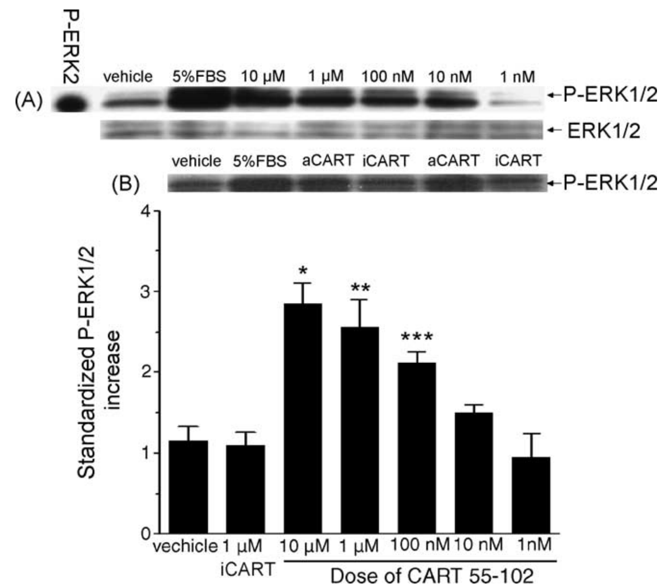

Fig. 3. Dose–response curve for phosphorylation of ERK1 and 2 in AtT20 cells treated with varying concentrations of active CART 55–102 (aCART) and response to 1 μM inactive CART 1–27 (iCART) after 4 min treatment. Representative Western blots are shown in panels A and B. The Western blot B represent the effects of 1 μM active CART 55–102 (aCART) and 1 μM inactive CART 1–27 (iCART) on ERK1 and 2 phosphorylation. The bar graphs reflect the quantification of the blots ($n = 4$). One-way ANOVA analysis revealed significant differences, $F_{(6,27)} = 12.22$, $p < 0.0001$. Tukey's test was used for post hoc analysis. $^{*}p < 0.001$, values at 10 μM were different from vehicle, iCART and 1 nM. Values at 10 μM were also different from 10 nM ($p < 0.001$). $^{**}p < 0.01$, values at 1 μM were different from vehicle and iCART. Values at 1 μM were also different from 1 nM ($p < 0.001$). $^{***}p < 0.005$, values were different from 1 nM.

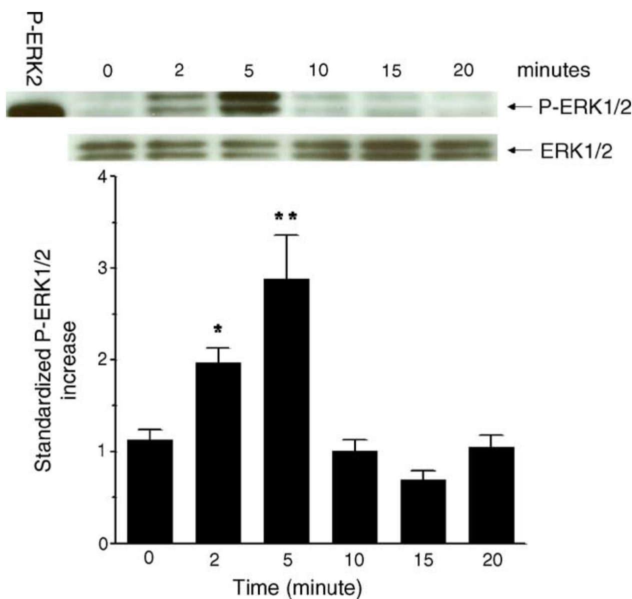

Fig. 2. The time course of P-ERK1 and 2 levels in AtT20 cells treated with 100 nM CART 55–102. Upper panel shows a representative Western blot for P-ERK and total ERK, with the position of a P-ERK2 standard on the left. Each blot shows distinct ERK1 and ERK2 bands. The bar graph below in the lower panel shows relative levels of P-ERK1 and 2 (mean ± S.E.) determined from the Western blots ($n = 4$). One-way ANOVA analysis indicates significant effects, $F_{(5,23)} = 13.36$, $p < 0.0001$. Tukey's test was used for post hoc analysis. Significantly different, $^{**}p < 0.001$, 5 min compared to 0, 10, 15 or 20. $^{*}p < 0.01$, 2 min compared to 15 min.

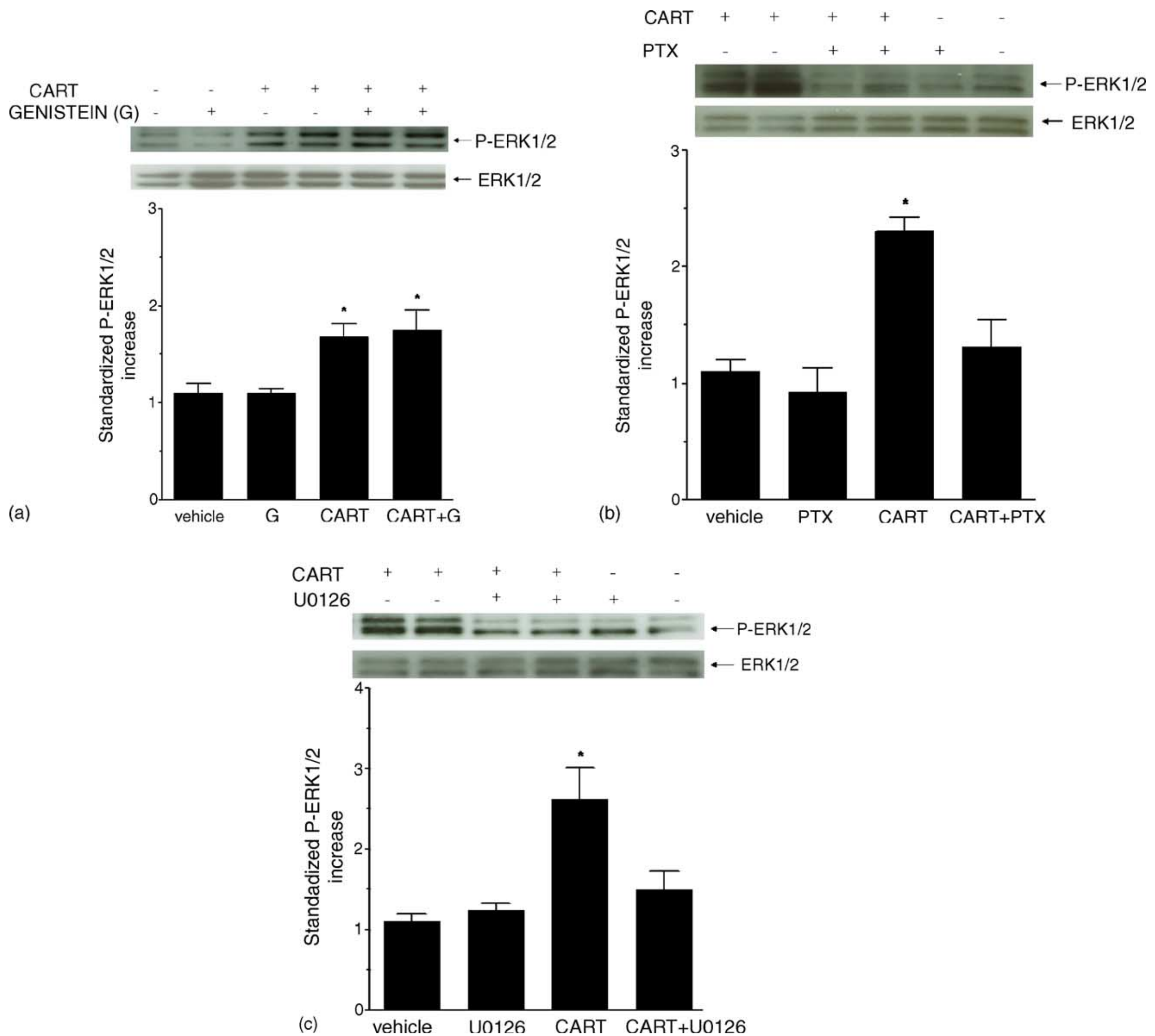

Fig. 4. Effects of genistein (G) (a), pertussis toxin (PTX) (b) and U0126 (c) on CART stimulated ERK phosphorylation in AtT20 cells. Cells were exposed to 10 μM U0126 for 30 min, or 200 ng/ml of PTX for 16 h, or 150 μM genistein (G) for 20 min or vehicle for the same times. 1 μM CART was added and the reaction terminated 4 min later. The top of each panel shows representative Western blots and the bar graph below shows relative levels of P-ERK1 and 2 ($n = 4$), determined. One-way ANOVA analysis revealed significant differences and Tukey's test was used for post hoc analysis. In (a), one-way ANOVA, $F_{(3,23)} = 7.05$, $p < 0.0015$; $^*p < 0.05$ for CART and CART + G different from vehicle and from G, but not from each other. In (b), one-way ANOVA, $F_{(3,23)} = 19.10$, $p < 0.0001$; $^*p < 0.001$ where CART is different from the other three values. In (c), one-way ANOVA, $F_{(3,23)} = 8.51$, $p < 0.0008$; $^*p < 0.01$ where CART is different from the other three values.

Accordingly we utilized inhibitors of GPCR- and RTKs-activated signaling pathways. Pertussis toxin, an inhibitor of Gi and Go signaling, and genistein, an inhibitor of RTK signaling, were tested for their effects on CART 55–102 induced ERK activation. Pertussis toxin effectively blocked ERK phosphorylation in response to CART stimulation (one-way ANOVA, $F_{(3,23)} = 19.10$, $p < 0.0001$) (Fig. 4b). Post hoc testing showed that CART's effects were significantly different from those of vehicle and CART + PTX ($p < 0.001$). While CART treatment increased P-ERK1/2 levels, pretreatment with genistein (G) did not alter ERK1/2 phosphorylation induced by CART (one-way ANOVA, $F_{(3,23)} = 7.05$, $p < 0.0015$) (Fig. 4a). Post hoc testing revealed that CART and CART + G were different from those of vehicle and G alone ($p < 0.05$), but not different from each other. To further elucidate the pathway by which CART peptide exert their effects on ERK activity, we examined the ability of U0126, an inhibitor of the MEK1 and MEK2 kinases [7], to block the actions of CART 55–102. One-way ANOVA revealed significant differences among the groups (one-way ANOVA, $F_{(3,23)} = 8.51$, $p < 0.0008$) (Fig. 4c). We found that 10 μM concentrations of U0126 completely blocked the effects of

CART on P-ERKs. Post host testing showed that CART's effects were different from those of vehicle, U0126 alone and CART + U0126 ($p < 0.05$). These data reveal that CART stimulation results in enhanced ERK activity via a pathway involving Gi/Go and MEK1 and 2.

Our findings show that CART 55–102 induces the activation of ERK1 and 2 in a time- and dose-dependent manner in AtT20 cells. The CART effect is blocked by U0126, indicating the involvement of the upstream kinases, MEK1 and 2. Pertussis toxin also inhibited the formation of P-ERK in response to CART treatment, suggesting the involvement of a Gi/Go coupled GPCR in CART signaling. Previous electrophysiological studies suggested that the effects of CART may involve G-proteins [17], but concrete biochemical evidence for a G-protein-mediated pathway activated by CART has been lacking. Our data strongly suggest that the CART receptor is a GPCR, specifically one that is coupled to Gi/Go. Interestingly, differences in the activation of ERK1 or 2 were observed which will be pursued in future studies.

ERK activation by CART peptide may underlie some of the physiological effects of CART peptide on cell growth and differentiation. As noted above, CART appears early in development and may be involved in the formation of various organs including the brain. In addition to potential actions in cell growth and differentiation [3], CART peptide has a variety of more established actions in the regulation of feeding and body weight [9], drug reward and reinforcement [10] and stress [1,15]. The role of CART in drug reward, reinforcement and addiction may also involve CART-mediated ERK activation.

No receptor for CART has yet been identified. Nevertheless, the specific effects found here suggest the existence of a CART-responsive GPCR that couples to Gi/Go and is capable of activating ERK. There is evidence that there may in fact be multiple CART receptors. For example, differences in relative potencies for CART 55–102 and CART 62–102 have been shown in food consumption [13,2], elevated plus maze activity [5], and hot plate antinociception, acoustic startle response, and prepulse inhibition [2]. These differences in potencies were not always of the same magnitude or in the same direction. This implies that there may be multiple signal transduction mechanisms activated by CART peptide, perhaps due to multiple receptor subtypes. Our data shown here elucidate a specific molecular pathway that is activated by CART peptides, revealing a functional assay that may be used to study CART-mediated signaling and eventually identify the CART receptor(s).

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