**Title:** BIOACTIVE PEPTIDES DERIVED FROM COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT PROTEIN

The present disclosure provides compositions and methods for modulating food consumption in an animal or human to which the compositions are administered. Peptides have specifically disclosed whose sequences increase food intake when administered in compositions comprising these peptides in combination with a pharmaceutically acceptable carrier or diluent.
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BIOACTIVE PEPTIDES DERIVED FROM COCAINE AND AMPHETAMINE
REGULATED TRANSCRIPT PROTEIN

CROSS REFERENCE TO RELATED APPLICATIONS
This application claims priority from United States Provisional Application No. 60/045,455, filed May 1, 1997.

ACKNOWLEDGEMENT OF FEDERAL RESEARCH SUPPORT
This invention was made, at least in part, with funding from the National Institutes of Health. Accordingly, the United States Government has certain rights in this invention.

THE BACKGROUND OF THE INVENTION
The invention relates generally to compositions and methods for regulatory behavior in a human or an animal via bioactive compounds, and, more particularly, to bioactive peptides (as specifically exemplified, peptides whose sequences are derived from the cocaine and amphetamine regulated transcript protein) and methods for suppressing appetite and food consumption, and methods for increasing food intake via administration of antibodies specific for peptides.

The cocaine and amphetamine regulated transcript encodes a polypeptide termed the cocaine and amphetamine regulated transcript protein (CART). High concentrations of the CART transcript are found in the nucleus accumbens of the brain. Transcription of the CART locus increases in the striatum after acute administration of psychostimulants.

Acute or chronic administration of drugs can alter gene expression in the brain. Understanding the changes can provide insights regarding addiction and also into mechanisms of other central nervous system active drugs such as antidepressants and antipsychotics. Cocaine administration is known to cause changes in immediate early genes (IEGs) as well as in a variety of other transcripts, proteins and neurotransmitters.

Amphetamine administration also alters IEG expression. Single doses of these drugs (cocaine


In addition to changes in known IEGs, there are also changes in a variety of mRNAs for proteins and peptides that occur after psychostimulant drug administration. cocaine or

CART is the third most abundant transcript in the rat hypothalamus after subtraction of cerebellar and hippocampal mRNAs [Gautvik et al. (1996) Proc. Natl. Acad. Sci. USA 93, 8733-8738]. It encodes a polypeptide of 116 or 129 amino acids in rat, depending on the (alternate) splicing of the mRNA. It is enriched in the nucleus accumbens, a site associated with the reinforcing effects of cocaine, and only neostriatal CART is elevated by acute cocaine administration. There is no significant homology of the CART nucleotide sequence to other sequences in Genbank. CART is localized in limbic regions, and it is found in circuitry associated with sexual function (the ventral premammillary nucleus of the hypothalamus and medial nucleus of amygdala), and with autonomic function (nucleus of the solitary tract and inferior olive). It is found in the locus coeruleus and ventrolateral medulla, suggesting that it is a peptide cotransmitter in catecholaminergic neurons. It is also found in the paraventricular nucleus of the hypothalamus and the pituitary and adrenal glands, suggesting a role in hypothalamus/pituitary/adrenal axis functions, such as stress and immune responses. Certain of these areas are involved in the control of feeding as well.
While most studies of CART have been in the rat, CART has a similar distribution in the human brain. There is 95% amino acid sequence identity between the human and rat polypeptides. The human CART gene is located on chromosome 5 [Douglass et al. (1995) J. Neuroscience 15, 2471-2481]. The sequences of the coding regions of human and rat CART, together with the deduced amino acid sequences of the encoded polypeptides, are presented in Tables 1 and 2.

There is a long felt need in the art for effective means and methods for the control of behavior, particularly with respect to appetite control and food consumption and modulating levels of physical activity, and also there exists a strong and long felt need in the art for means and methods for treating addictive behavioral disorders.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide compositions and methods for the control of the behavior of a human or an animal. Compositions of the present invention are useful in suppression of appetite and thus, reduction of food consumption, and/or for increasing the level of physical activity of a human or animal to whom the composition has been administered. The present invention further provides means and methods for control of addictive disorders in humans and in animals. As specifically exemplified, a peptide of the amino acid sequence IPIYEKKYGQVPMCDAGEQCAV (SEQ ID NO:1), which corresponds in sequence to amino acids 82-103 of the rat CART polypeptide, and a peptide of the amino acid sequence VPIYEKKYGQVPMCDAGEQCAV (SEQ ID NO:2), which corresponds in sequence to amino acids 82-103 of the human CART polypeptide, suppress appetite. A peptide derived therefrom, having the amino acid sequence YGQVPMCDAGEQCAV (SEQ ID NO:3) (corresponding in sequence to amino acids 89-103 of the CART polypeptides of rat and human) suppresses appetite without a concomitant increase in the level of locomotor activity. Within the scope of the present invention are pharmacological compositions comprising at least one peptide as set forth above and a pharmaceutically acceptable carrier. Within the scope of the term peptide are peptides with structural modifications which do not significantly affect the biological activity when compared with the peptide composed solely of L-amino acid residues. Peptides which have identical sequences to those specifically exemplified with substitutions of D-amino acid
residues for the corresponding L-amino acid residues, especially at one or more non-terminal positions, are within the scope of the present invention. Also within the scope of the present invention are peptides comprising the recited sequences, or their functional equivalents, which have been acylated or myristoylated. Similarly, substitution of a methionine residue with a norleucine residue can improve peptide stability. Generally structural modifications such as those set forth herein act to increase the in vivo half-life of the peptides, and thus, enhance the net effectiveness of the peptides. One or more modifications of the amino acid sequence of a peptide of the present invention is within the scope of present Applicants' invention. Also within the scope of the present invention are methods for treating addictive disorders using one or more of the peptides (or their functionally equivalent modified forms) of the present invention.

The present invention further provides antibody preparation (polyclonal and/or monoclonal) with binding specificities for CART-derived peptides. CART-derived peptides include those having amino acid sequences including, but not limited to, those corresponding to 82-103, 89-103 and 106-129 of the human or rat CART polypeptide (SEQ ID NO:7; full-length). Also within the scope of the present invention are those antibody preparations which specifically recognize peptides derived in sequence from the human or rat CART polypeptide.

Also within the scope of the present invention is a method for increasing food intake in an animal or human via the administration, directly or indirectly, to the brain in a dosage effective for the desired effect, of an antibody preparation with specificity for the rat and human CART-derived peptides corresponding in sequence to amino acids 82-103 SEQ ID NO:1 and SEQ ID NO:2, respectively. Also within the scope of the present invention is a method for decreasing the locomotor stimulation associated with cocaine or amphetamine administration, abuse or addiction, said method comprising the step of administering to the brain, directly or indirectly, an effective amount of an antibody preparation with binding specificity for an epitope within a peptide having an amino acid sequence corresponding in sequence to amino acid residues 106-129 of the rat CART polypeptide (SEQ ID NO:17). The present invention further provides for treatment of Attention Deficit Hyperactivity Disorder via administration of compositions comprising antibodies specific for antagonists or blockers of a peptide as in SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.
BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show the effect of icv injection of CART peptide fragments on dark phase food intake in the rat. Fig. 1A: Effects of rat CART peptides 82-86 (SEQ ID NO:4), 82-103 and 89-103 (4 nmol/5 µl; icv) on dark-phase food intake (g/2 hr) compared to 0.9% saline (sal) (n=5-13). Fig. 1B: Dose response curve for CART 82-103 (0.4, 4, 40 nmol/5 µl; icv) on dark-phase food intake (g/2 hr) (n=7). See hereinbelow for details. Statistical analysis of data shown in Figs. 1A and 1B was by paired t-test. * - indicates statistically significant differences from sal group. p<0.05.

Figures 2A-2B show the effects of icv injection of preimmune (pre) and immune serum (C3Ab) to CART 82-103 on dark-phase food intake (g/2 hr). Fig. 2A: icv injection of preimmune (5 µl) and immune (5 µl) serum (n=11). Fig. 2B: Volume-response to icv injection of immune serum (1, 3, 10 µl) compared to preimmune serum (pre. 3 µl) (n=4). Statistical analysis of data shown in Fig. 2A was paired t-test. * - indicates a statistically significant difference from pre group, p<0.05. Statistical analysis of data shown in Fig. 2B was by one way repeated measures ANOVA (F(3,13) = 21.8. p<0.001) followed by a post hoc Tukey test. P<0.05 compared to pre group.

Figure 3 illustrates the effects of a five minute pretreatment with 0.9% saline (sal. 5 µl; icv) or CART 82-103 (4 nmol/5 µl; icv) on NPY-induced food intake (g/2 hr) in the rate (n=6). Statistical analysis of data was by paired t-test. ** - indicates a statistically significant difference from sal/NPY group, p<0.01.

Figure 4 shows the effect of icv injection of preimmune serum (PI) or an antibody against CART peptide 106-129 (C4Ab) on locomotor activity in the first hour following an injection of cocaine (10mg/kg, ip.). The antisera was administered 15 minutes before the injection of cocaine. The experiment was conducted in the dark phase in a group of six male Sprague-Dawley rats. * indicates a significant difference by paired t test from the PI+coc group, p<0.05.

Figure 5 shows the effect of icv injection of saline, preimmune serum or an antibody against CART peptide 106-129 (C4Ab) on locomotor activity. The data is shown in 30 min periods (0-30, 30-60) for one hour following icv injection. The experiment was conducted in the dark phase in a group of six male Sprague-Dawley rats. * indicates a significant difference
by paired t test between the first and second 30 minute periods of locomotor activity following injection of C4Ab, p<0.05.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are given in order to provide clarity as to the intent or scope of their usage in the specification and claims.

CART is a cocaine and amphetamine regulated transcript. Sequences of the human and rat CARTs, together with the deduced amino acid sequences of the encoded polypeptides, are provided in Tables 1 and 2, respectively. WO 96/34619, which is incorporated by reference in its entirety, also describes the human genomic CART sequence.

As used herein, a CART polypeptide (SEQ ID NO:9) is encoded by the human CART mRNA, as disclosed in SEQ ID NO:8 (desirably this sequence) herein. It is noted that the rat CART message is alternatively spliced. The rat cDNA sequence is given in SEQ ID NO:6. The full-length (129) sequence is provided in SEQ ID NO:7, the shorter (116) (SEQ ID NO:10) alternate form lacks the internal amino acid sequence PRRQLRAPFAVLQ (SEQ ID NO:16). See also SEQ ID NO:7 and SEQ ID NO:10 for the preferred rat alternative deduced amino acid sequences.

A non-naturally occurring recombinant nucleic acid molecule, e.g., a recombinant DNA molecule, is one which does not occur in nature; i.e., it is produced either by natural processes, directed by the skilled artisan using methods known to the art, and directed by man to produce a desired result or it has been artificially produced from parts derived from heterologous sources, which parts may be naturally occurring or chemically synthesized molecules or portions thereof, and wherein those parts have been joined by ligation or other means known to the art.

The term an effective amount as used herein refers to the amount of bioactive peptide which is sufficient to cause a significant effect on behavior (food consumption and/or physical activity) when administered to an animal or human in a pharmaceutically acceptable formulation. Dosages giving the desired degree of effectiveness are readily ascertained by art-known methods.

A coding sequence used in a DNA construct of this invention may be modified, if desired, to create mutants, either by random or controlled mutagenesis, using methods known
to those skilled in the art. Such mutants and variants are therefore within the scope of the present invention. Accordingly, the phrase "polypeptide" as used herein includes native or synthetic proteins, truncated proteins and fusion proteins, capable of affecting behavior in the animal or human to which it has been administered or in which it has been expressed. Where a polypeptide is administered or expressed, there can be proteolytic processing of the polypeptide to release two or more peptides, which in turn may have biological activity(ies).

In one embodiment of the present invention, a cDNA sequence that encodes human or rat CART can be genetically engineered for expression in a particular target site, such as brain or other organ or tissue, or it can be engineered for constitutive expression.

Which CART peptide fragments should be studied? The CART peptide has 5 pairs of basic amino acids. Three of these are frequent or preferred sites (KR, RR) for cleavage while the other two are more rarely used. Processing at the preferred sites results in CART peptides 28-54, 57-79, and 92-129. The 82-129 fragment is difficult to synthesize because it is long and because of the many cysteines and the potential for disulfide bond formation.

Immunoreactivity for CART peptide fragments 28-54, 57-79, 82-103 and 106-129 was found to be present in brain, including hypothalamic areas [Koylu et al. (1997) Neuroendocrinology 9, 823-833; Smith et al. (1997) Synapse 27, 90-94]. CART fragments comprising amino acids 82-111 and 82-93 were found in extracts of ovine hypothalamus by Spiess [Spiess et al. (1981) Biochemistry 20, 1982-1988], but only partial sequencing of the fragments was carried out and the full sequence of extracted fragments remains undetermined. Accordingly, fragments that included these sequences and other related fragments were chosen for study.

We have made additional subfragments which possess activity at receptors for the large 82-129 fragment, e.g., the peptides corresponding in amino acid sequence to CART amino acids 82-103, 89-103 and 106-129. All of these fragments occur between pairs of basic amino acids. Subfragments of active neuropeptides can be antagonists or can be modified to produce antagonists [for example, Schiller et al. (1994) Reg. Peptides 54(1), 257-258; Schmidt et al. (1996) Int. J. Peptide Protein Res. 48(5), 411-419; Hernandez et al. (1993) J. Med. Chem. 36(20), 2860-2867; Boden et al. (1993) J. Med. Chem. 36(5), 552-565].

Many kinds of modification of peptides have been made [for example, Bouvier and Taylor (1992) J. Med. Chem. 35(6), 1145-1155; Kapurniotu and Taylor (1995) J. Med. Chem. 38(5),
836-847]. Other peptides have been shown to affect motor behavior and operant responding in the mesocorticollimbic pathway [for example, Kelley et al. (1985) Psychopharmacology 85(1), 37-46; Kelley et al. (1989) Psychopharmacology 97(2), 243-252; Robledo et al. (1993) Brain Res. 622(1-2), 105-112].

Because there is one pair of basic amino acids in the 82-103 fragment, we subdivided it at this site and studied the effect of the subfragments as well: 82-86 and 89-103. The large fragment, 89-103, produced a reduction in food intake (Table 1). This effect is shared by psychostimulant drugs. We tested the two subfragments at equimolar doses (82-86 and 89-103) that could result from the cleavage. 89-103 reduced food intake but had no effect on locomotor activity. The strength of the peptides' effects (but not the positive v. negative effects) on a particular behavior depend, at least somewhat, however, on whether measurements are made in the light or dark phase: this kind of effect has been seen before with peptides [e.g., Davies and Wellman (1990) Pharmacol. Biochem. Behav. 37, 201-203; Tempel and Leibowitz (1989) Brain Res. Bull. 23(6), 553-560].

Moreover, since the parent or complete CART polypeptide has five pairs of basic amino acids and is likely to be processed by proteases [Spiess et al. (1981) Biochemistry 20, 1982-1988], it is essential that we have the ability to detect different fragments. Accordingly, we have developed five separate polyclonal antisera to five distinct peptide fragments. Four of the fragments are distinct possible products of processing. Antibodies to the following fragments have been made: 24-39; 29-54 (between leader sequence and first pair of basic amino acids); 57-79 (between first and second pairs of basic amino acids); 82-103 (between second and fourth pairs); and 106-129 (between fourth pair of basic amino acids and end).

All of these antibodies clearly show specific immunoreactivity. The specificity of the immunoreactivity is supported by the observation that cells expressing CART mRNA, as determined by in situ hybridization studies, also stain with antisera specific for the CART-derived peptides, and the observation that the immunostaining is blocked by the immunizing peptides but not by irrelevant peptides of these antibodies has allowed us to extensively map portions of the colchicine-treated and colchicine-untreated rat brain at the light microscopic level (hypothalamus, pituitary and adrenals, primate nucleus accumbens). That CART mRNA is found in high levels in cells in the shell of the nucleus accumbens in the rat supports our thesis that CART peptides are involved in psychostimulant drug action. Light
microscopic immunohistochemistry also shows that CART peptides are in the shell of the accumbens.

Because of potential species differences, it is important for us to establish that CART is also found in the primate nucleus accumbens. Experiments (immunolocalization techniques) showed that CART peptides were localized in the nucleus accumbens of the nonhuman primate. There is a high degree of amino acid sequence identity between the rat and human CART amino acid sequences. Compare SEQ ID NO:7 and SEQ ID NO:9. We note that the human coding sequence does not appear to produce a 129 amino acid CART polypeptide.

Our results clearly show that CART peptides are found in elevated levels in the shell of the nucleus accumbens of primates (squirrel and rhesus monkeys were tested), indicating that previous work in the rat applies to primates. Further, electron microscopic immunohistochemistry in the primate accumbens shows that CART peptides are found in nerve terminals and in large dense core vesicles, which supports our thesis that CART peptides are neurotransmitters. It also indicates that CART peptides can be cotransmitters in GABAergic neurons in the accumbens. We have found CART in some ganglion cells of the retina, in mitral cells in the olfactory bulb, in lamina X in parts of the spinal cord, in the anterior pituitary and in the adrenal medulla.

The experiments described herein identify CART peptides that have inhibitory effects on food intake following central injection. Considering that the effect is an inhibition, there is a concern that it may be nonspecific. Another concern is that since the endogenously occurring peptides have not been fully sequenced and identified, it isn’t known whether these effects of the CART 82-103 peptide reflect the activity of the endogenous peptides at their putative receptors. Both concerns were addressed in the following experiments where the effects on food intake of icv injection of antipeptide antisera were examined. In these immunoneutralization experiments, central activity of endogenous CART peptides is inhibited. Polyclonal antisera raised against the CART 82-103 peptide produced a volume dependent (1, 3, 5, 10 μl) increase in food intake after icv injection compared to injection of pre-immune sera (Figs. 3A, 3B). The finding that the icv effect of the antisera is stimulatory and opposite to the effects of the peptides indicates that the observed effects are not nonspecific and reflect the activity of endogenous CART peptides. Further, the stimulation
caused by the antisera suggest that CART peptides exert an inhibitory tone on feeding, at least in the first two hours of the dark phase.

Neuropeptide Y (NPY) is the most powerful stimulant of feeding known [Clark et al. (1984) *Endocrinology* 115, 427-429; Lambert et al. (1993) *Endocrinology* 133, 29-32] and has been shown to functionally interact with other hypothalamic neuropeptides, such as dynorphin [Lambert et al. (1993) *Brain Research* 629, 146-148] and GLP-1 7-36NH₂ [Turton et al. (1996) *Nature* 379, 69-72] in the central control of food intake. CART peptide-immunoreactivity is found in hypothalamic nuclei containing NPY [Koylu et al. (1997) supra], and it is generally accepted that central satiety factors interact with the NPY system to achieve fine control of feeding behavior. Therefore, we sought to determine both functional and anatomical interactions between CART peptides and the NPY system. NPY is well established as a powerful orexigenic agent, and we show that icv injection of NPY (2.4 nmol/5 µl) dramatically increased two-hourly food intake (10 ± 1 g) compared to injection of saline (1.0 ± 0.4 g) (Fig. 4). Interestingly, icv injection of CART 82-103 (4 n mmol/5 µl) 5 minutes before injection of NPY (2.4 nmol/5 µl) significantly attenuated the feeding response (7.7 ± 1.1 g) (Fig. 4). These studies were also of crossover design with all animals receiving all treatments on study days 48 hours apart.

Having demonstrated a functional interaction between CART 82-103 and NPY, we conducted immunohistochemical studies in the rat paraventricular nucleus of the hypothalamus (PVN), at the light microscopic level, to test if there is an anatomical interaction between NPY and CART peptide-immunoreactive elements. Immunohistochemical studies in the rat paraventricular nucleus of the hypothalamus show that CART peptide-positive bodies and NPY-positive elements are in apposition.

Injection of antibodies specific to a peptide comprising the amino acid sequence of SEQ ID NO:17 or antagonists of a peptide as in SEQ ID NO 17 reduces cocaine-induced locomotor activity (Figure 4) indicates that CART peptides, antibodies or blockers can be used in the treatment of substance abuse.

In summary, the data presented herein identify CART peptides as novel central components in the control of food intake and indicate a role for endogenous CART peptides as physiological satiety factors. The apparent interaction with NPY indicates that CART peptides are involved in the known hypothalamic circuitry that regulates feeding.
Our experiments have been carried out using injection into the lateral ventricles, and the anatomical sites of action are determined. Cannulae are implanted into the following brain regions and directly injected with 2-3 of the most potent CART peptides: the nucleus accumbens, the sites where neurons in the nucleus accumbens project; the VTA, ventral pallidum, medial substantia nigra, and lateral hypothalamus [Pennartz et al. (1994) Prog. Neurobiol. 42, 719-761]. Anorectic effects are determined similarly. Groups (n = 6-8) will be administered peptides or saline and food intake will be measured as described below. Once the minimal dose for producing an effect is identified, additional doses are tested to provide and measure dose response effects.

In addition to the effects of CART-derived peptides on feeding, the present invention further provides compositions and methods for modulating the endocrine system as reflected by peripheral hormone levels. Inhibition of CART synthesis via icv administration of antisense oligonucleotides directed against CART message or icv administration of neutralizing antibodies specific for the CART 82-103 peptide (SEQ ID NO:1) results in decreased serum testosterone. The repeated icv administration of antibodies specific for the peptide of SEQ ID NO:1 resulted in a decrease in serum corticosterone as well as a significant decrease in body weight. Without wishing to be bound by theory, it is believed that the weight loss is due to an increase in the peripheral metabolic rate as a result of the icv immunoneutralization of the 82-103 CART peptide (SEQ ID NO:1).

While many of the experiments described herein relate to animal experiments, the skilled artisan knows how to formulate the bioactive peptides of the present invention for administration by other routes, such as intramuscular, intravenous and the like. A desirable formulation is one in which the peptide is incorporated within liposomes which can cross the blood-brain barrier, such as liposome formulations, are well within the skill in the art. See, WO 96/34619 for a discussion of formulations and methods of administration (incorporated by reference herein in its entirety).

In another embodiment, polyclonal and/or monoclonal antibodies capable of specifically binding to the full-length CART polypeptide or to a peptide corresponding in sequence to a portion thereof, are provided. The term antibody is used to refer both to a homogenous molecular entity, or a mixture such as a serum product made up of a plurality of different molecular entities. Monoclonal or polyclonal antibodies which specifically react
with the peptides of the present invention may be made by methods known in the art. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratories; Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, New York. Also, recombinant immunoglobulins may be produced by methods known in the art, including but not limited to, the methods described in U.S. Patent No. 4,816,567. Monoclonal antibodies with affinities of \(10^5\) M\(^{-1}\), preferably \(10^9\) to \(10^{10}\) or more, are preferred.

Antibodies specific for the CART polypeptide or a peptide corresponding in amino acid sequence to a peptide derived therefrom are useful, for example, as probes for screening DNA expression libraries or for detecting the presence of the cognate polypeptide or peptide in a test sample. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or noncovalently, a substance which provides a detectable signal. Suitable labels include but are not limited to radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. United States Patents describing the use of such labels include but are not limited to Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.


All references cited in the present application are incorporated by reference herein.

The following examples are provided for illustrative purposes, and are not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified sequences and methods which occur to the skilled artisan are intended to fall within the scope of the present invention.

EXEMPLARY EXAMPLES

**Example 1. Peptide synthesis**

The CART derived peptides are prepared by Merrifield's solid phase peptide synthesis methodology using a peptide synthesizer (Beckman model 990) as described [Ling et al. (1984) *Proc. Natl. Acad. Sci. USA* 81, 4302-4306]. The t-butyloxy carbonyl protected amino acid derivatives and the resin used in the synthesis were purchased from Bachem (Torrance, CA). In brief, the synthetic procedure involved repeating a series of consecutive steps, which consisted of deprotection of the blocked N-terminal amino function on the peptide chain with 50% trifluoroacetic acid, neutralization of the deprotected N-terminal residue with 10% diisopropylethylamine and coupling of the next protected amino acid to the free N-terminal of the growing peptide chain with dicyclohexyl carbodiimide.

After the last protected amino acid was coupled onto the peptide chain, the protected peptide-resin was treated with 10% anisole in hydrogen fluoride to remove the peptide from the resin anchor as well as to deprotect the side-chain functional groups on the assembled peptide. The recovered crude peptide was purified by preparative HPLC using a Kiloprep 100 gradient HPLC system (Biotage, Charlottesville, VA) and the purified peptides were characterized by analytical HPLC and mass spectrometry.
Example 2. Methodology for ICV injections for behavioral experiments.

Peptides (Neurocrine Biosciences Inc., San Diego, CA. USA: Multiple Peptide Systems, San Diego, CA. USA) were dissolved in 0.9% saline.

Male rats (Sprague-Dawley), average body weight 300g, are housed singly under a controlled reverse light-dark cycle (12h each phase, lights off 10 am) in a temperature controlled environment. Food and water are available ad libitum. Rats are anesthetized with an injection of ketamine (Ketalar, 100 mg/ml, 0.2 ml ip) followed by a single injection of sodium pentobarbital (Nembutal, 50 mg/ml; 0.2 ml). Once anesthesia has been established, the rat's head is fixed in a stereotaxic frame and a single guide cannulae (22 gauge) is implanted just above the lateral ventricle (AP + 0.8, L 1.4, V -3.0). Three stainless steel screws are attached to the skull and the cannula is fixed in place using dental cement applied around the screws and the cannula. A 26 gauge stainless steel flush-fitting stylet is inserted into the cannula to prevent blockage. Animals are allowed at least five days to recover from surgery before being used in the experimental procedure. Infusions are made in conscious rats in their home cages. The stylet is removed and a 26 gauge steel camula projecting 1 mm below the guide cannula is inserted. The injection cannula is connected via tubing (Intramedic PEIO polyethylene tubing; Clay Adams) to a 10 μl Hamilton syringe driven by a Hamilton apparatus 22 infusion pump. The syringe and tubing are filled with sterile 0.9% NaCl, and a small air bubble is drawn up into its distal end. The bubble separates the infused solution from the saline and also acts as a convenient index of a successful infusion. Two μl of the test solution is infused at a speed of 2.5 μl/min, and the infusion cannula is left in place for a further minute immediately following infusion. The animal is placed in the test arena, and its locomotor activity and/or food intake is measured for the following 2 hr. The rat's locomotor activity (distance moved, time spent moving and rearing) is measured using a Noldus Ethovision Videotracking and Motion Analysis system. The test arena consists of an empty wooden box (height 24", width 30", and depth 30") with an open top. Animals are acclimatized to the arena for an hour a day for the two days prior to their first test day. On the day of the experiment, they are placed in the box for thirty minutes prior to ICV injection and then returned to the arena for a two hour test period. During this time their movement is recorded using a monochrome video camera, and images are digitized and analyzed using the motion analysis software. The data are analyzed by time in order to determine the onset and
duration of action of the peptide. A two hr test period is chosen because the effects of most compounds injected directly into the brain begin within 10 minutes of injection and are completed within 2 hr.

For the measurement of food intake, acclimatized rats are placed in the test arena along with a known amount of standard rat chow pellets and free access to water. At the end of the two hour test period, all remaining food is collected and reweighed to determine total food intake. During these experiments, the rats' behaviors are also recorded to determine if effects seen on food intake are specific or due to other competing behaviors. All locomotor activity and food intake experiments are carried out in the dark phase. The laboratory rat is nocturnal and, therefore, its baseline activities and any changes made to that are best monitored in the dark. All experiments are of a crossover design with each rat receiving an ICV injection of peptide followed by saline or vice versa. A period of at least 48 hr is allowed between test days and no animal receives more than one drug treatment. The data show that, following a single injection of peptide, an animal's locomotor activity or food intake can be altered such that assessment of the effects of other fragments could not be determined in the same animal. All data from both activity and food intake studies are grouped by treatment; mean and standard errors of the mean are calculated, and significant differences between groups are assessed using appropriate statistical methods.

Example 3. Biological Activity of CART-derived Peptides

Male Sprague-Dawley rats were implanted with cannulae in their cerebral lateral ventricles by surgical procedures, allowed to recover and divided into groups of 3-5 rats each. Each of the four groups received one of three peptides (having amino acid sequences corresponding to amino acids 82-86, 82-103 or 89-103 of the rat CART polypeptide (with reference to SEQ ID NO:7) or a saline control injection (by intracerebral ventricular injection, icv) immediately prior to lights out. Dark phase food intake, measured in grams, was measured over the next 2 hrs. The dosing was repeated in the remaining four groups, but following ICV injection of a peptide composition or a saline control, food was withdrawn and locomotor activity (DM = distance moved) in the first 2 hours of dark phase using a video tracking system. In some experiments, locomotor activity was measured in the light phase as well. The results are shown in Table 1.
The results presented in Table 1 are the first evidence for any biological activity of any peptide or polypeptide product of the CART. The peptide corresponding in sequence to amino acids 82-103 of the CART-encoded polypeptide exhibited anorectic activity. This effect is consistent with psychostimulant activity typical of cocaine or amphetamine administration. The 89-103 CART-derived peptide inhibits feeding. These results are in contrast to other biologically active peptides, such as NPY, which stimulate feeding [Ludnell et al. (1995) J. Biol. Chem. 270(49), 29123-29128; Kirby et al. (1995) J. Med. Chem. 38(22), 4579-4586; Weng et al. (1995) Molec. Pharmacol. 48(1), 9-14].

Immunohistochemical studies show high densities of CART peptide-containing neurons in brain regions mediating feeding. It is interesting that CART 89-103 inhibits feeding. At least some peptides known to stimulate feeding, such as NPY, inhibit adenylate cyclase [Lundell et al. (1995) J. Biol. Chem. 270(49), 29123-29128; Kirby et al. (1995) J. Med. Chem. 38(22), 4579-4586; Weng et al. (1995) Molec. Pharmacol. 48(1), 9-14].

Because antibodies to CART peptides, for example peptides comprising an amino acid sequence as given in SEQ ID NO:17 or SEQ ID NOS: 1, 2 or 3, reduce locomotor activity (Figures 5, 6), CART peptides can affect or modulate the action of psychostimulant drugs indicating a role in treating substance abuse or Attention Deficit Hyperactivity Disorder (ADHD), respectively. Administration of antibodies or antagonists specific for a peptide with a sequence as given in SEQ ID NO:17 is an effective treatment for substance abuse or addiction, including without limitation, cocaine or amphetamine addiction. Administration of antibodies specific for peptides having an amino acid sequence as given in SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or antagonists of those peptides improve behavior in an ADHD patient by reducing locomotor activity.

CART peptides therefore have some psychostimulant effects and/or modify the effects of psychostimulant drugs. Our data clearly indicate that CART peptides have some psychostimulant-like effects upon direct injection into the lateral ventricles. These effects include an anorectic action.
Example 4. Depletion of CART peptides by administration of antisense oligonucleotides.

If CART peptides are neurotransmitters or neuromodulators in circuitry related to psychostimulant drug action, then removal of active CART peptides themselves has an effect, and also should affect the response to cocaine. Accordingly, we examine the effects of immunoneutralization and the administration of antisense oligonucleotides on the cocaine dose-response curve and time course of action in locomotor activity experiments.

Our experiments show that administration of antisense oligonucleotides into the nucleus accumbens bilaterally depressed the locomotor response of rats to cocaine. However, we were unable to achieve statistically significant differences. We also inject oligonucleotides and antibodies into the lateral ventricles so that more sites of action are affected. Certain parts of the accumbens may be more important than others for CART's action, and the most effective location within the accumbens for the cannulae is determined. Also, for the immunoneutralization experiments, because the important sites of CART action could be at the sites of projection of the CART neurons from the accumbens, it is important to inject antibodies into the ventricles to reach these more distant sites of action. Thus, the strategy is to inject oligonucleotides and antibodies into the ventricles to affect more sites rather to inject them into a specific site in the nucleus accumbens. Successful antisense experiments have been published [Sommer et al. (1993) Neuroreport 5, 277-280; Wahlestedt et al. (1993) Science 259, 528-531; Zhang and Creese (1993) Neurosci. Let. 161(2), 223-226; Rossi et al. (1994) Life Sciences 54(21), PL375-379]. Immunoneutralization has been effective in showing the physiologic role of some peptides [see, e.g., Le Feuvre et al. (1991) Brain Res. 555(2), 245-250; Wehrenberg et al. (1989) Neuroendocrinology 49(6), 680-682; Lambert et al. (1993) Endocrinology 133, 29-32; Sarnyai et al. (1992) Brain Res. 589(1), 154-156].

Locomotor activity is measured in four groups of animals (n= 9- 10) at a time; 3 different groups receive three different types of oligonucleotides (see below) and the last group receives vehicle alone. Locomotor activity is measured in groups without psychostimulants and also in groups given psychostimulants. In the former case, we test the effects of oligos alone on locomotor activity and in the latter case, we determine if the oligos affect cocaine-induced locomotor activity.
In animals given cocaine, we measure EC50 values which will reflect potency, the maximal or peak activity which reflects efficacy, and the duration of action of the drug effects. The antisense oligos could affect any of these three parameters.

Male Sprague-Dawley rats are injected with 1, 3, 10 or 30 mg/kg of cocaine and locomotor activity is measured over two hours. Comparisons are made among the four groups.

Cocaine and oligonucleotide effects on locomotor activity are determined in male rats as described above. The procedure is as follows:

Rats are placed in the test chambers individually between 10.00 and 11:00 a.m. They are given 60 min to habituate, then injected i.p. with cocaine in a volume of 0.1 ml/100 g of body weight. Beginning 5 min later, activity is recorded for a total of 2-3 hours. In some experiments cocaine is tested and retested periodically in order to detect equipment problems or seasonal affects on response to the drug.

The software has the capability to measure numerous variables, such as time in motion, distance traveled, and time spent in the center of the chamber, and some of those parameters can prove useful for distinguishing among compounds. Dose-response curves are constructed for each compound in two ways: 1) area under the time-effect curve, determined by the trapezoidal rule, 2) peak effect, based upon a single 15 min interval. Standard statistical procedures (e.g., two-factor analysis of variance and linear regression analysis) are used to compare the groups with respect to a) duration of action (vs. vehicle control); b) relative potency; and c) relative efficacy (with the maximum effect of cocaine designated 1.00).

Antisense, missense and sense oligonucleotides are made and to correspond to numbered bases in parentheses as described in Douglass et al. (1995) *J. Neuroscience* 15, 2471-2481. The sequences of the oligonucleotides are as follows:

antisense (1-19) 5'-TGGTGCTGGACTTCCTCGCT-3' (SEQ ID NO:11);
(101-120) 5'-TGCAGCTCGCATGTGGTG-3' (SEQ ID NO:12);
(130-149) 5'-CGGCCAGAGTATGTCCAGG-3' (SEQ ID NO:13);
misssence 5'-CGGCAGAGAAGTGTCAGCAG-3' (SEQ ID NO:14);
sense 5'-AGCGAGGAAGTCCAGCACA-3' (SEQ ID NO:15). Three kinds of oligos are used: unmodified phosphodiester backbone; and 5' and 3' thioester end capped. The latter have a longer half-life and are preferred.

The first schedule of oligo injection is as follows: (50 μg in 5 μl injection icv, twice a day for 5 days). In another schedule, oligos are infused via minipumps continuously through cannulae into the lateral ventricles at 1 μl/hr at 5 μg/ml for 3-5 days [Wahlestedt (1994) Trends in Pharmacol. 15, 42-46; Whitesell et al. (1993) Proc. Natl. Acad. Sci. USA 90, 4665-4669]. After treatments, locomotor activity is measured. The animals receiving drug are habituated to the chambers for 60 min, given cocaine i.p., and placed in the chambers for an additional 120 min. Important control experiments are as follows. Where changes due to antisense treatment are found, we test for reversibility of these changes by stopping oligo injection but continuing to measure locomotor activity. Reversibility is important to rule out toxic, irreversible neuronal damage. Also, where the effects are due to antisense caused depletion of CART peptides, then missense or sense have no effects. Immunohistochemistry is used to assess changes in peptide levels and detect tissue damage.

Icv injection of a mixture of antisense oligos to CART (having nucleotide sequences as given in SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:13; 75 μg), once a day for five days, had no significant effect on daily body weight or food intake compared to injection of missense oligo or saline. Interestingly, however, serum testosterone at the end of the study was significantly decreased in the antisense group (by approximately 60%) compared to both missense and saline groups. Serum corticosterone, growth hormone and insulin were unchanged by this treatment.

**Example 5. Immunoneutralization Experiments**

Three groups of rats are utilized: the first group is saline injected, the second is given preimmune serum, and the third is given immune serum. In different experiments we use separate sera directed at least three different portions of the CART peptide. Injections will be made into the lateral ventricles by procedures already described. Each of these three groups are explored in rats with no pretreatment, and in rats given cocaine. Pretreatment with cocaine is useful in these experiments because where psychostimulant drugs cause a release of CART peptides which in turn mediate some of the effects of the drug, it is instructive to
give the antibodies under conditions where the naturally occurring peptides are active, i.e., when the animals have received drugs. Immunoneutralization of CART peptides is studied in animals without drugs and in animals given psychostimulant drugs. Varying dilutions of antibodies are given to obtain a dose/dilution that causes effects. Five \( \mu l \) of undiluted sera is generally an effective dose [Lambert et al. (1993) Endocrinology 133, 29-32]. The time when the antibodies are given in relationship to the time cocaine is given is also varied: antibody 30 min - 1 hr before, immediately after, and some time after the administration of cocaine. Locomotor activity and dosing with cocaine is carried out as described above.

If antisense oligonucleotides, but not missense or sense oligonucleotides, alter the locomotor activity effects of cocaine, then we conclude that CART peptides can influence or mediate cocaine's actions. Similarly, if immunoneutralization with antisera directed against specific peptides, but not preimmune sera, alters cocaine's effects on locomotor activity, then we conclude that CART peptides can affect the effects of psychostimulant drugs.

Antibodies to individual CART-derived peptides are tested as follows. Rats are cannulated icv and allowed to recover as described hereinabove. A 5 \( \mu l \) volume of peptide-specific antibody (or 5 \( \mu l \) preimmune serum as control) is injected icv to determine the effect of immunoneutralization in an animal which has not received a psychostimulant. Dark phase food intake is monitored over a 2 hr period. An antibody preparation with specificity for CART 8'-103 caused a statistically significant increase in food intake. Surprisingly there was no apparent effect on locomotion activity in the rats treated with the 8'-103 immune serum.

In a second experiment, 5 \( \mu l \) antiserum collected after immunization with a peptide corresponding in sequence to CART 106-129 (SEQ ID NO:17)(or 5 \( \mu l \) preimmune serum) was administered icv 15 min. prior to intraperitoneal cocaine injection. Dark phase locomotion activity was significantly reduced in the rats treated with immune serum (see Figure 4). Feeding was not monitored in this experiment. Locomotor activity was also reduced by injection of the antiserum (raised to CART 106-127) in rats to whom cocaine was not administered.

**Example 6. Anorectic Activity of CART Peptides.**

Since psychostimulant drugs have anorectic effects, and since CART peptides are involved in effects of psychostimulant drugs, we carried out experiments to demonstrate that
CART-derived peptides have anorectic effects. CART mRNA and peptides are found in high abundance in the hypothalamic areas involved in feeding. These include the paraventricular nucleus, the supraoptic nucleus, perifornical cells and the arcuate nucleus.


Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Food Intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% saline</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>CART 82-103, 10 μgm</td>
<td>3.2 ± 0.2*</td>
</tr>
<tr>
<td>CART 82-86, 2.5 μgm</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>CART 89-103, 6.5 μgm</td>
<td>2.6 ± 0.3*</td>
</tr>
</tbody>
</table>

The peptides listed under above are used in these experiments. Groups of rats (n = 6-8) receive intraventricular injections of the various peptides or vehicle.

Initially, various CART peptides were injected in equimolar quantities, and food intake in the first two hours of the dark phase was measured (Fig. 2A). Experiments were of crossover design with each animal receiving a peptide treatment and saline on separate days 48 hours apart. CART 82-103 (4 nmol/5 μl) and 89-103 (4 nmol/5 μl) reduced food intake by 26 and 40% respectively compared to injection of saline (5 μl). lev injection of 0.4, 4, and 40 nmol of CART 82-103 resulted in a dose-dependent inhibition of food intake (Fig. 2B).

For CART peptides having anorectic activity, it is believed that the anorectic effects of these peptides is through hypothalamic feeding mechanisms, at least in part. The fact that
CART peptides are found in neurons and neural cell groups in the hypothalamus involved in feeding and the fact that CART peptides are found in the same neurons in the hypothalamus where other anorectic or feeding altering peptides are found, indicates that CART peptides exert their effects through hypothalamic mechanisms.

We also found in immunoneutralization experiments that injections of CART peptide-specific antibodies enhance feeding.

**Example 7. Distribution and Levels of CART peptides**

Using specific antibodies to CART peptide fragments to map the brain of rat, monkey and human by immunohistochemistry, and to localize CART mRNA by *in situ* in these species plus the mouse.

Cocaine is known to alter a variety of peptides in brain [see, e.g., Kosofsky et al. (1994a) *J. Comp. Neurol.* 351, 27-40; Kosofsky et al. (1994b) *J. Comp. Neurol.* 351, 41-50; Cole et al. (1995) *Neuron* 14, 813-823; Levy et al. (1991) *J. Pharmacol. Exper. Ther.* 259(2), 495-500; Levy et al. (1992) *Pharmacol. Biochem. Behav.* 42(3), 481-486]. Where CART peptides play a role in psychostimulant drug action, then are in neuronal circuits affected by these drugs. Where CART peptides function as neurotransmitters or neuromodulators, then they are localized to the same neurons that contain the CART mRNA rather than being expressed constitutively throughout the brain. In addition, one expects to find CART peptide immunoreactivity in axons and nerve terminals.

We have raised five separate antibodies to several distinct CART peptide fragments, demonstrated specific immunoreactivity with all (five) of these peptides tested so far and showed that CART peptide immunoreactivity is found in neurons that were previously reported to contain CART mRNA. Moreover, by carrying out peptide mapping experiments with different antibodies to different CART peptide fragments, one obtains an indication of CART peptide processing in various neuronal populations in brain.

The CART gene has been identified in humans and shows a very high level of sequence homology with rat [Douglass et al. (1995) *J. Neuroscience* 15, 2471-2481], and our data show CART peptides are highly elevated in the nucleus accumbens of monkey just as in the rat. In situ hybridization studies are carried out in mouse brain; this is relevant to "knockout" studies (see below).
Anatomical mapping is carried out in 5-8 male, Sprague-Dawley rats, 3-4 rhesus monkeys, and 5-8 mice (strain compatible with studies of "knockout" mice). Both immunohistochemistry and in situ hybridization are performed as described below. Observations are consistently made in at least 3 animals.

The fragments for which we have generated antibodies are: 24-39 (a fragment following the putative leader sequence, 28-54 (following the putative leader sequence up to first pair of basic amino acids), 57-79 (between first and third pair of basic amino acids), 92-103 (between third and fifth pair of basic amino acids; 106-129 (between last pair of basic amino acids and end). All references to amino acid residue numbers are with respect to the rat CART polypeptide of Table 3 unless otherwise specified.

The antibodies were prepared by standard procedures in rabbits. Each antibody is effective in immunocytochemistry at a 1:10,000 dilution. Each antibody is blocked by the immunizing peptide but not by the other four peptides. Serial sections processed with different sera allows the obtention of evidence for differential CART processing in different neurons.

Example 8. Light Microscopic Immunohistochemistry Procedures.

Animals are perfused with buffered 4% paraformaldehyde. After perfusion, the brains are removed from the skulls and placed in perfusion buffer for 5 hrs at 40°C, and then in buffered sucrose (10, 20, and 30%) for 24 hrs each. The brains are then stored at -80°C. 40 micron thick sections are prepared in a cryostat. Human tissue is obtained from the Department of Psychiatry brain bank.

Sections are washed in Tris buffered saline (TBS) for 10 min, washed in sodium borohydride (1% in TBS) for 10 min, washed twice in TBS for 10 min each time, and incubated with 4% normal goat serum (NGS), 1% BSA, and 0.4% Triton X-100 for blocking. The sections are then incubated with primary antibody overnight at 4°C in a buffer containing Triton X-100 (0.4%) NGS (1%) and BSA (1%). The next day, the sections are brought to room temperature for 2.5 hr in the primary antibody incubation mixture. The sections are then washed 10 times in mixtures of TBS, 0.25% BSA and 0.02% Triton-X-100 for 10 min. Following the 10 washes, the sections are incubated with the secondary antibody at a concentration of 1.33 μg/ml for 60 min at room temperature in a buffer containing 1% BSA.
and 0.02% Triton X-100. The sections are then washed 4 times for 15 min each at room temperature in TBS containing 0.25% BSA. The sections are then incubated with the ABC complex in 1% BSA (the ABC complex is prepared 30 min before incubation with sections). The sections are then washed in imidazole acetate buffer twice for 10 min each and developed in DAB solution for 2-10 min at room temperature. Following development, the sections are washed in imidazole acetate buffer twice for 10 min at room temperature, washed in TBS twice for 10 min at room temperature, and then mounted onto slides. After air drying, the sections are coverslipped. All of the incubation and wash steps are carried out on an orbital shaker.

Electron microscopic immunohistochemistry has been carried out. The results show that CART peptides are in neurons, nerve terminals and dense core vesicles in the nucleus accumbens. The cytochemistry suggests that these neurons may be GABAergic projection neurons. At least some of these neurons in the accumbens are known to have dopaminergic input [Voorn et al. (1996) J. Comp. Neurol. 251(1), 84-99; Pickel et al. (1988) J. Comp. Neurol. 272, 1-14; Sesack and Pickel (1990) Brain Res. 527, 266-279; Trotterdell et al. (1989) J. Chem. Neuroanat. 2, 285-298].

These experiments dramatically demonstrate that CART peptides are found in precisely the same neurons that contain CART mRNA. We have shown that CART peptides are highly enriched in the nucleus accumbens of primates as well as in rats, observations in keeping with an important role for CART peptides in psychostimulant drug action in primates.

In order to examine whether there is an anatomical interaction in the rat paraventricular nucleus of the hypothalamus (PVN) between NPY and CART peptide-immunoreactive elements, rat brain sections at the level of the PVN were incubated first with a sheep anti-NPY antiserum (1:500; Chemicon International, Temecula, CA) for 48 hours at 4°C. NPY immunoreactivity was then revealed with the ABC method using DAB as the chromogen. The CART peptide immunoreactivity was localized second using Ni-DAB [for more details see Wouterlood et al. (1987) J. Histochem. Cytochem. 35, 817-823; Koylu et al. (1997) Neuroendocrinology 9, 823-833; Smith et al. (1997) Synapse 27, 90-94]. In double immunostained sections, NPY-containing varicosities were found to form dense pericellular baskets around the perikarya of CART peptide-immunoreactive neurons in the parvicellular
region of the PVN (Figs. 4B, 4C). Electron microscopic studies allow confirmation that NPY-immunoreactive terminals establish direct synaptic contacts with CART peptide-containing neurons in the rat and monkey PVN. The data show CART peptide-positive cell bodies and NPY-positive elements in apposition.

Example 9. CART Northern Blot Analyses.

Total RNA is extracted from tissue with a modified acid phenol-guanidine isothiocyanate protocol. Seven μg total RNA is run on 1.2% agarose-6% formaldehyde gel; the resulting gel is washed in water and blotted onto a Nytran membrane with 2OX SSC. Blots are hybridized 14-16 hrs at 60°C with a cRNA probe (CART, nucleotides 42-800, 1B15 or cyclophilin, nucleotides 35-685) in hybridization buffer (5% SDS, 400 mM phosphate buffer, pH 7, 1 mM EDTA, 1 mg/ml BSA (Fraction V) and 50% formamide). The blots are subsequently washed for 15 min at room temperature, 70°C for 1 hr. and 70°C for 3 hrs with changes of wash solution (1% SDS, 0.05X SSC and 1 mM EDTA) at each time point. Moist blots are then placed moist between Saran wrap and exposed to Kodak BIOMAX MR film.

Example 10. In situ hybridization procedure.

Fresh-frozen brains are sectioned at 20 μm thickness and thawed mounted onto gelatin/poly-L-lysine coated slides and stored at -70°C. Slides are post-fixed in 4% paraformaldehyde, positive charges blocked with acetic anhydride, dehydrated, defatted and rehydrated. 35S-labeled cRNA probe is mixed with hybridization solution (50% formamide, 0.3 M NaCl, 10 mM TRIS (pH 8.0), 1 mM EDTA, 50 mM DTT, 1X Denhardt’s, 500 μg/ml yeast tRNA. 500 μg/ml polyA RNA and 10% Dextran), applied to each section (2 x 10^6 cpm/section) and covered with coverslip. Sections are hybridized 14-16 hours in a humidifying chamber at 55°C. Post-hybridization washing consists of 2X SSC (5'), 2X SSC (15')· 2X SSC + 20 μg/ml RNase A + β-mercaptethanol (30' at 37°C), 2X SSC (15'), H2O (5 brief dips) then dehydrated to 95% ethanol. Sections are then air dried and exposed to Kodak BIOMAX MR film. Following that, slides are dipped in Kodak NBT-2 emulsion, stored at 4°C, and developed 2-4 weeks thereafter.

When using film, regional densities are quantified using image analysis and radioactive standards [Kuhar and Unnerstall (1990) Receptor Autoradiography. In: Methods
in Neurotransmitter Receptor Analysis. H.I. Yamamura et al. (Eds.) Raven Press, Ltd., New York, pp. 177-218]. When studying emulsin-dipped sections, grain densities are quantified by counting using a light microscope as carried out with dopamine transporter mRNA [Cerruti et al. (1993) Mol. Brain Res. 18, 181-186; Cerruti et al. (1994) Mol. Brain Res. 22, 132-138].

We have tested for postmortem changes in CART at early times in rats. We could not detect any changes in CART levels when brains were allowed to remain at room temperature postmortem for up to 5 min. There were no changes in CART in tissue stored frozen for 1 month. Thus, postmortem changes are not an issue in these experiments because there is ample time to freeze the tissues after dissection.

Radioimmunoassays are developed against various CART fragments. The fragments 28-54 and 82-103 have tyrosine residues to permit iodination. Fragments 57-79 and 106-129 are prepared with N-terminal tyrosines added. Thus, we can detect segments along the entire CART chain. The antibodies are all effective in immunocytochemistry, at least at the dilution of 1:10,000.

Example 11. CART Fusion Protein Expression in vitro.

Both alternatively spliced rat CART variants were subcloned into pET expression vectors (Novagen, Madison, WI) and expressed as histidine tag bacterial fusion proteins. The putative protein coding regions (amino acids 28-116 and amino acids 28-103 of the rat CART polypeptide), excluding the N-terminal 27 amino acids, were subcloned into pET-23b (long CART). Proteins were expressed in the E. coli 13L21 (DE3) pLysS strain.

For bacterial expression of fusion proteins, a single bacterial colony was grown overnight at 37°C in 3 ml LB broth with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). A 100 µl aliquot of an overnight culture was transferred into 100 ml LB broth with antibiotics. Cell growth was followed until an OD₆₀₀ of 0.4 - 0.6 was obtained. Protein expression was induced by adding IPTG (1 mM final concentration) and incubating for an additional 3 hr. Cells were subsequently harvested by centrifugation and the cell pellet stored at -80°C. Bacterial aliquots were removed at different time intervals to monitor protein expression by SDS-PAGE and Western blot analysis.
Bacterial-expressed fusion proteins were affinity-purified with a Ni-NTA agarose column (Qiagen, Chatsworth, CA). Frozen cell pellets were resuspended in ice cold 40 ml binding buffer [5 mM imidazole, 0.5 M NaCl, 20 mM TRIS (pH 7.5), 5 mM beta-mercaptoethanol] and sonicated (5 x 30 sec) while on ice. Lysates were centrifuged at 39,000 x g for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 ml binding buffer, sonicated and pelleted as previously described. This pellet was resuspended in 10 ml binding buffer and 6 M urea and chilled on ice for 60 min. This suspension was centrifuged at 39,000 x g for 20 min at 4°C. The supernatant was isolated and filtered through a 0.45 μm filter. This filtered lysate was applied to Ni-NTA agarose previously equilibrated as follows. A 2 ml Ni-NTA agarose sample was mixed with 2 ml H₂O, poured into a column and allowed to settle. The water was eluted and 10 ml H₂O was passed over the column. The column was then equilibrated for 30 min with 10 ml binding buffer with 6M urea. The filtered cell lysate was added to the column and allowed to mix on a shaker for 60 min at room temperature. The resin was then allowed to settle and washed 3 x 5 ml of binding buffer with 6 M urea and 3 x 7 ml wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris (pH 7.5), 5 mM beta-mercaptoethanol, 6M urea). The proteins were eluted with 5 ml elution buffer [300 mM imidazole, 0.5 M NaCl, 20 mM Tris (pH 7.5), 5 mM beta-mercaptoethanol, 6 M urea]. The eluate was dialyzed 2 x 45 min in 1 L 0.5 M NaCl, 20 mM Tris (pH 6.5). 0.1% Triton X-100 with decreasing concentrations of urea (4 M, 2 M, 1 M, 0.5 M and 0 M). A final dialysis for 1 hr in 0.5 M NaCl, 20 mM Tris (pH 6.5), 0.1% Triton X-100, 0.5% glycerol with 2 changes of buffer followed. The resulting dialysate was lyophilized. Aliquots of samples were taken during the protein purification protocol for analysis of protein purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Example 12. CART Knockouts.

The most common method for generating knockout mice is deletion or interruption of the gene of interest through homologous recombination. For these purposes, the mouse CART gene is required for established procedures. The rat and human cDNAs are 82% identical and 90% homologous within the coding region. A rat cDNA probe is used to isolate the mouse gene based on sequence similarities.
Approximately $1 \times 10^6$ clones from a mouse 129 SV/J genomic library are plated on 20 plates and screened with a rat cDNA random primer probe that spans the entire coding region. Two filters lifted from each plate are hybridized in 50% formamide, 400 mM NaPO$_4$, (pH 7.4), 1 mM EDTA, 1 mg/ml BSA, 100 $\mu$g/ml sheared salmon sperm DNA with radiolabelled probe for 14-16 hr at 37°C. Filters are washed in 2X SSC, 1% SDS and 1 mM EDTA at room temperature for 30 min followed by a change of wash solution and a wash at 45°C for 2-3 hrs. At least 6 hybridization positive plaques as determined by the presence of a signal from both filters are isolated. These plaques are subjected to two further rounds of purification and selection to insure purity and are then physically mapped with restriction endonucleases, and sequenced from both strands. Clones are cycle sequenced by walking from each end of the DNA insert starting with vector-directed primers and then clone-specific primers until both strands are read.

A 2 kb clone from a human lambda Charon4A library was found to contain the entire human CART gene with approximately 100 bases of the 5’ untranslated region. We can therefore expect a 2 kb or larger clone; a clone with several hundred bases of either 5’ and 3’ untranslated regions is desirable. Because the cDNA sequences for the rat and human genes are available, confirmation of the mouse gene sequence is straightforward.


The mouse gene clone is amenable to modification without further subcloning. A cassette containing the neomycin resistance and at least 5 kb of the DNA sequence containing the CART gene is constructed to interrupt the mouse CART gene near the translation initiation site in exon 1. A herpes simplex virus thymidine kinase gene is positioned at the 5’-prime end of the entire construct to allow negative selection with FIAU. The construct is
sequenced in both directions for verification. Vector sequences are removed before the introduction of the construct into embryonic cells. The vector is designed so that a homologous recombination event generates an interrupted CART gene by the insertion of a neomycin resistance in exon 1. The inserted genes allow for selection embryonic stem cells carrying the disrupted CART gene with G418 and FIAU. The location of the cassette within the CART translation initiation site disrupts any potential alternatively spliced mouse CART mRNA variants. The generation of a knockout mouse is straightforward and follow procedures well known to the art.

Vertebrate animals used in this project include mice, rats and monkeys. The rats (male, Sprague-Dawley) are purchased from standard sources and handled in accordance with AAALAC guidelines. Some of the animals are killed within one or two weeks of arrival and serve as a source of fresh tissue. Some rats are treated surgically; implantation of chronic indwelling cannulas is carried out by standard approved procedures. The monkeys are squirrel monkeys (*Saimiri sciureus*) obtained from standard commercial sources or from the existing population at the Yerkes Primate Center, Emory University. They are sacrificed by approved procedures, and the brains are dissected and frozen for histochemical analysis. These protocols have been approved by the Emory University Institutional Animal Care and Use Committee.
(1) GENERAL INFORMATION:

(i) APPLICANT: EMORY UNIVERSITY
    KUHAR, Michael J.
    LAMBERT, Philip D.
    COUCEYRO, Pastor R.

(ii) TITLE OF INVENTION: Bioactive Peptides Derived from Cocaine and Amphetamine Regulated Transcript Protein

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
    (A) ADDRESSEE: Greenlee, Winner and Sullivan, P.C.
    (B) STREET: 5370 Manhattan Circle, Suite 201
    (C) CITY: Boulder
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    (E) COUNTRY: US
    (F) ZIP: 80303

(v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Floppy disk
    (B) COMPUTER: IBM PC compatible
    (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
    (A) APPLICATION NUMBER: WO
    (B) FILING DATE: 01-MAY-1998
    (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
    (A) APPLICATION NUMBER: US 60/045,455
    (B) FILING DATE: 01-MAY-1997
(viii) ATTORNEY/AGENT INFORMATION:
   (A) NAME: Ferber, Donna M.
   (B) REGISTRATION NUMBER: 33,878
   (C) REFERENCE/DOCKET NUMBER: 26-97 WO

(ix) TELECOMMUNICATION INFORMATION:
   (A) TELEPHONE: (303) 499-8080
   (B) TELEFAX: (303) 499-8089

(2) INFORMATION FOR SEQ ID NO:1:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 22 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: not relevant

   (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

   Ile Pro Ile Tyr Glu Lys Lys Tyr Gly Gln Val Pro Met Cys Asp Ala
   1     5     10     15

   Gly Glu Gln Cys Ala Val
   20

(2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 22 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: not relevant
(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Pro Ile Tyr Glu Lys Lys Tyr Gly Gln Val Pro Met Cys Asp Ala
  1   5    10   15
Gly Glu Gln Cys Ala Val
        20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

  (A) LENGTH: 15 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

15

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Tyr Gly Gln Val Pro Met Cys Asp Ala Gly Glu Gln Cys Ala Val
  1   5    10   15

(2) INFORMATION FOR SEQ ID NO:4:

20

(i) SEQUENCE CHARACTERISTICS:

  (A) LENGTH: 5 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: not relevant
(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ile Pro Ile Tyr Glu

5

|i| 1 | 5 |

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 14 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Gly Glu Val Pro Met Cys Asp Ala Gly Glu Gln Cys Ala

15

|i| 1 | 5 | 10 |

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 840 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Rat

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCGAGGAAAG TCCAGCACCA TGGAGAGTTC CCGCCTGCCG CTGTACCCCG TCCTGGGCCG 60
5
CCGCCCTACTG CTGCTGCTAC CTTTGCTGGG TGCCGGTGCC CAGGAGGATG CCGAGCTGCA 120
GCCCGGAGCC CTGGACACCT ACTCTGCGGT GGATGATGCC TCCCCATGAGA AGGAGCTGCC 180
AAGGCGGCAA CTTCCGGCCTG CCGCGCCTGT GTTSCAGATTT GAAGCGCTGC AGGAACTCTT 240
GAAGAAGCTC AAGAGTAAAC GCATTCCGAT CTATGAGAAG AAGTACGGCC AGTGCCCCAT 300
GTGTGACGCT GGAGAGCAGT GCGCAGTGCG GAAAGGGGCCC AGGATCGGGA AGCTGTGTA 360
10
CTGTCCCCGA GGAACCTCTTT GCAAATTCTTT CCTCTTTGAA TGCTTTGAGA GGGGTGACAG 420
CCTCCTTCGG TTCCCCATATT TCCTCTTCCC CCTAAAGGAG CGCTCTTTTT CCTCTGGAGC 480
CGCTTTAACA ACAATAAAGT TTGCGTCCC CCCAGAGAGT GGATGGGCTC TTTCCTGCT 540
GCTTCAAAAT AAAAAATTTG ATGTTTTTGT GTGAAGGACA ATACCTTGAA TGCTTTGCTT 600
ATGTGTGCAAG AGTAATTCTTC TCTCCTTTTA TCCACCTGAC AATTTCTGTG GACCTTTCTG 660
15
GGAAGAAGAG GGACTTTCGC TTTAAAACCTG TATTTTTGTA TGCTGGCGGT CACAATGAAG 720
ATTAGACCTA GTTAATTGGA GCAGATGACA TCATAACCGG GAAACAAAT CACCCCCAAG 780
CAACACAAT GGAAGCATGT GCAAATTACA CCCAAAAG CATTTTTGAT ATGTGTCTCA 840

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rat

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:?

Met Glu Ser Ser Arg Leu Arg Leu Leu Pro Val Leu Gly Ala Ala Leu
1  5  10  15
Leu Leu Leu Leu Pro Leu Leu Gly Ala Gly Ala Gln Glu Asp Ala Glu
  20  25  30
Leu Gln Pro Arg Ala Leu Asp Ile Tyr Ser Ala Val Asp Asp Ala Ser
  35  40  45
His Glu Lys Glu Leu Pro Arg Arg Gln Leu Arg Ala Pro Gly Ala Val
  50  55  60
Leu Gln Ile Glu Ala Leu Gln Glu Val Leu Lys Leu Lys Ser Lys
  65  70  75  80
Arg Ile Pro Ile Tyr Glu Lys Tyr Gly Gln Val Pro Met Cys Asp
  85  90  95
Ala Gly Glu Gln Cys Ala Val Arg Lys Gly Ala Arg Ile Gly Lys Leu
100 105 110
Cys Asp Cys Pro Arg Gly Thr Ser Cys Asn Ser Phe Leu Leu Lys Cys
115 120 125
Leu
(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 800 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
    (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AACGACCGAGT TTCAGAACGA TGGAGAGCTC CCAGGTAGGG CTGCTGCCCC TCCTGGCGGC  
   60
CGCCCTGCTG CTGATGCTAC CTCTGTTGGG TACCCGTGCC CAGGAGGAGG CCGAGCTCCA  
   120
GCCCCGAGCC CTGGACATCT ACTCTGCCGT GGATGATGCC TCCACGGAGA AGGAGCTGAT  
   180
CGAAGCGCTG CAAGAAGCTCT TGAAGAAGCT CAAGAGTAAA CGTGTTCCTA TCTATGAGAA  
   240
GAAGTAGTGGC CAAGTCCCCA TGTGTGACGC CGGTGAGCAG GGTGCAGTGA GGAAGGGGC  
   300
AAGGATCGGG AAGCTGTTGG ACTGTCGCCG AGGAAACCTC TGCAATCTCT TCTCTCTGAA  
   360
GTGCTTATGA AGGGCGTCC ATTCTCTCTC ATACATCCCC ATCCCCCTAC TTTCCCCCGA  
   420
GGACCACACC TTTCTCTCTG GAGTTTGCGT TAAGCAACAG ATAAAGTTTT TATTTTCTTC  
   480
TGAAGGGAAA GGGCTCTTTT CCTGCTGTTT CAAAAATAAA AGAAACACATT AGATGTTACT  
   540
GTGTGAAAGAA TAATGGCTTT TATGGTTGGT ATACGTGTTGT GAAGTATTCT TATTTTATT  
   600
GTCTGACAAA CTCTTGTGTA CCTTTGTGTA AAGAAGGGA ACGTTGTGTTT AAAATTGTAT 660
TTTTGTATGT GCCATGGCAG AATGAAAATT AGATCTAGCT AATCTCGGTA GATGTCATTA 720
CAACCTGGAA AATAAATCAC CCTAAGTGAC ACAAAATTGAA GCATGTACAA ATTATACATA 780
ATAAAGTGTT TTTAATAATT 800

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Glu Ser Ser Arg Val Arg Leu Leu Leu Gly Ala Ala Leu 1 5 10 15

Leu Leu Met Leu Pro Leu Leu Gly Thr Arg Ala Gln Glu Asp Ala Glu 20 25 30

Leu Gln Pro Arg Ala Leu Asp Ile Tyr Ser Ala Val Asp Asp Ala Ser 35 40 45

His Glu Lys Glu Leu Ile Glu Ala Leu Gln Glu Val Leu Lys Lys Leu 50 55 60
39
Lys Ser Lys Arg Val Pro Ile Tyr Glu Lys Tyr Gly Gln Val Pro
65 70 75 80
Met Cys Asp Ala Gly Glu Gln Cys Ala Val Arg Lys Gly Ala Arg Ile
85 90 95

Gly Lys Leu Cys Asp Cys Pro Arg Gly Thr Ser Cys Asn Ser Phe Leu
100 105 110
Leu Lys Cys Leu
115

(2) INFORMATION FOR SEQ ID NO:10:

10 (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 116 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: not relevant

15 (ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Rat

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Glu Ser Ser Arg Leu Arg Leu Leu Pro Val Leu Gly Ala Ala Leu
20 1 5 10 15

Leu Leu Leu Leu Pro Leu Leu Gly Ala Gly Ala Gln Glu Asp Ala Glu
25 30

Leu Gln Pro Arg Ala Leu Asp Ile Tyr Ser Ala Val Asp Asp Ala Ser
35 40 45
His Glu Lys Glu Leu Ile Glu Ala Leu Gln Glu Val Leu Lys Lys Leu
50                   55                   60
Lys Ser Lys Arg Ile Pro Ile Tyr Glu Lys Tyr Gly Gln Val Pro
65                   70                   75                   80
Met Cys Asp Ala Gly Glu Gln Cys Ala Val Arg Lys Gly Ala Arg Ile
85                   90                   95
Gly Lys Leu Cys Asp Cys Pro Arg Gly Thr Ser Cys Asn Ser Phe Leu
100                  105                  110

Leu Lys Cys Leu
115

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGTTGCTGGA CTTCTCGCT

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGCAGCTCGG CATGGGTTG

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGGCAGAGTA GATGTCCAGG

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGGCAGAGAA GTTGTGCACG

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCGAGGAAG TCCAGCACCA

(2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 13 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Arg Arg Gln Leu Arg Ala Pro Phe Ala Val Leu Gln

1      5     10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 24 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gly Ala Arg Ile Gly Lys Leu Cys Asp Cys Pro Arg Gly Thr Ser Cys

20    1     5     10  15

Asn Ser Phe Leu Leu Lys Cys Leu

20
WE CLAIM:

1. A peptide having an amino acid sequence substantially identical to a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:17.

2. A composition comprising at least one peptide of claim 1 and a pharmaceutically acceptable carrier or diluent.

3. A method for producing an anorectic response in an animal or human, said method comprising the step of administering to an animal or a human a composition in an amount effective for decreasing food consumption by said animal or human, wherein the composition comprises a peptide having an amino acid sequence as given in SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3.

4. The method of claim 3 wherein the step of administering is intracranial administration.

5. The method of claim 3 or claim 4 wherein the human or animal has a disorder selected from the group comprising obesity, type II diabetes and Prader-Willi syndrome.

6. A method for regulating body weight levels in an animal or human, wherein the animal or human has a disorder selected from the group comprising anorexia, bulimia or cachexia, said method comprising the step of administering an antagonist or an antibody specific for a peptide having an amino acid sequence as given in SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 in an amount effective for increasing food consumption in the animal or human to which the antibody or antagonist has been administered.

7. A method for regulating locomotor activity in an animal or human, wherein the animal or human has Attention Deficit Hyperactivity Disorder, said method comprising the
step of administering an antagonist or an antibody specific for a peptide having an amino acid sequence as given in SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 in an amount effective for decreasing locomotor activity in the animal or human to which the antibody or antagonist has been administered.

8. A method for treating substance abuse, addiction or acute administration of a substance which is cocaine or an amphetamine in an animal or human, said method comprising the step of administering an antagonist or an antibody specific for a peptide having an amino acid sequence as given in SEQ ID NO:17 in an amount effective for reducing the locomotor stimulation in the animal or human to which the antibody or antagonist has been administered.

9. A transgenic mouse in which function of the gene encoding a CART polypeptide has been inactivated.
FIG. 1A

Food intake (g/2 hr)

<table>
<thead>
<tr>
<th>Treatment (4 nmol in 5 ul, icv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sal</td>
</tr>
<tr>
<td>82-86</td>
</tr>
<tr>
<td>82-103</td>
</tr>
<tr>
<td>89-103</td>
</tr>
</tbody>
</table>

FIG. 1B

Food intake (g/2 hr)

<table>
<thead>
<tr>
<th>Treatment (nmol, icv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sal</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>40</td>
</tr>
</tbody>
</table>

SUBSTITUTE SHEET (RULE 26)
FIG. 2A

FIG. 2B
FIG. 3

FIG. 4
FIG. 5
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6): A61K 38/00, 38/02; C07K 5/00, 7/00, 9/00.
US Cl.: Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.: 514/12, 13, 14, 15, 16, 17; 530/324, 325, 326, 327, 328, 329, 330.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

APS, CA, BIOSIS, MEDLINE, EMBASE, MARPAT.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>US 5,413,779 A (KUHAR et al.) 09 May 1995, see entire document.</td>
<td>1-9</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search

22 JUNE 1998

Date of mailing of the international search report

15 JUL 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks

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Authorized Officer
AVIS M. DAVENPORT
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)
A. CLASSIFICATION OF SUBJECT MATTER:
US CL :
514/12, 13, 14, 15, 16, 17; 530/324, 325, 326, 327, 328, 329, 330.