The novel neuropeptide cocaine–amphetamine-regulated transcript (CART) is expressed in several hypothalamic regions and has recently been shown to be involved in the central control of food intake. To characterize the hypothalamic CART neurons and understand the physiological functions they might serve, we undertook an in situ hybridization and immunohistochemical study to examine distribution and neurochemical phenotype of these neurons. In situ hybridization studies showed abundant CART mRNA in the periventricular nucleus (PeV), the paraventricular nucleus of the hypothalamus (PVN), the supraoptic nucleus (SON), the arcuate nucleus (Arc), the zona incerta, and the lateral hypothalamic area. The distribution of CART-immunoreactive neurons as revealed by a monoclonal antibody raised against CART(41–89) displayed complete overlap with CART mRNA. Double immunohistochemistry showed co-existence of CART immunoreactivity (CART-IR) and somatostatin in some neurons of the PeV. In the magnocellular division of the PVN as well as the SON, CART-IR was demonstrated in both oxytocinergic and vasopressinergic perikarya. In the medial parvocellular region of the PVN a few CART-IR neurons co-localized galanin, but none was found to co-localize corticotopin-releasing hormone. In the Arc, almost all pro-opiomelanocortinergic neurons were shown to contain CART, whereas no co-localization of CART with NPY was found. In the lateral hypothalamic area nearly all CART neurons were found to contain melanin-concentrating hormone. The present data support a role for CART in neuroendocrine regulation. Most interestingly, CART is co-stored with neurotransmitters having both positive (melanin-concentrating hormone) as well as a negative (pro-opiomelanocortin) effect on food intake and energy balance.

Key words: cocaine–amphetamine-regulated transcript; CART; POMC; MCH; orexin; leptin; NPY; CRH; somatostatin; galanin; vasopressin; oxytocin; food intake; feeding behavior

The hypothalamus is a key player in controlling endocrine, autonomic, and behavioral aspects of homeostasis through its widespread reciprocal connections to forebrain and hindbrain sensory and motor systems and limbic areas (Swanson, 1987). The understanding of these functions has been greatly advanced during the last decades with the discovery of numerous neuropeptides, some of which are produced by distinct subgroups of neurons within the hypothalamus. The distribution of the different neuropeptides and their possible co-storage within neurons have been used as a guide to unravel the function and connectivity of the individual hypothalamic subnuclei.

One such recently discovered neuropeptide is cocaine–amphetamine-regulated transcript (CART). CART mRNA was originally identified by differential display techniques as a transcript acutely upregulated in rat striatum after cocaine and amphetamine administration (Douglass et al., 1995). However, CART mRNA is abundantly expressed in untreated animals in both forebrain and hindbrain as well as in several hypothalamic nuclei (Douglass et al., 1995), further emphasized by the observation that CART mRNA is among the most abundant of expressed hypothalamic mRNAs (Gautvik et al., 1996). The distribution of CART peptide immunoreactivity in the hypothalamus has been mapped using antibodies generated against synthetic fragments of CART (Koylu et al., 1997, 1998) or a CART fusion protein (Kristensen et al., 1998) and has shown CART immunoreactivity in approximately the same areas that have been described to contain CART mRNA.

CART is synthesized by neurons in several hypothalamic nuclei known to be involved in regulation of food intake, and we have recently shown that recombinant CART(42–89) inhibits food intake (Kristensen et al., 1998; Vrang et al., 1998). Also, we have shown that the population of CART neurons residing within the hypothalamic arcuate nucleus (Arc) are sensitive to the energy balance of the animal, in that fasting reduces the expression of CART mRNA (Kristensen et al., 1998). In fa/fa rats and ob/ob mice CART mRNA is virtually absent from the arcuate nucleus whereas no co-localization of CART with NPY was found. In the lateral hypothalamic area nearly all CART neurons were found to contain melanin-concentrating hormone. The present data support a role for CART in neuroendocrine regulation. Most interestingly, CART is co-stored with neurotransmitters having both positive (melanin-concentrating hormone) as well as a negative (pro-opiomelanocortin) effect on food intake and energy balance.

Key words: cocaine–amphetamine-regulated transcript; CART; POMC; MCH; orexin; leptin; NPY; CRH; somatostatin; galanin; vasopressin; oxytocin; food intake; feeding behavior
The widespread expression of CART mRNA within the hypothalamus suggests that CART peptide could play a role in regulating other functions besides feeding behavior. To characterize further the role of CART peptide in the hypothalamic neuronal circuitry, we undertook a series of experiments to clarify the anatomical distribution of CART mRNA as well as CART immunoreactivity within the hypothalamus. Subsequently dual-labeling immunohistochemistry was performed to unravel phenotypic characteristics of hypothalamic CART neurons. Major emphasis was placed on characterization of co-existence with neurotransmitters previously implicated in neuroendocrine regulation as well as control of feeding behavior.

MATERIALS AND METHODS

Animals and tissue preparation. Adult male Wistar rats (200–300 gm) were used for both the immunohistochemistry and the in situ hybridization studies.

In situ hybridization. Rats were decapitated, and the brains were rapidly removed and frozen on dry ice. Twelve-micrometer-thick frontal sections were cut on a freezing microtome and mounted directly on Superfrost Plus slides. In situ hybridization analysis was performed (Kristensen et al., 1991) on cryostat sections using antisense RNA probes directed against the rat CART cDNA (bp 226–411; GenBank accession number U10771). Posthybridization washes were performed at 62 and 67°C in 50% formamide. After hybridization, sections were exposed on β-Max film (Amersham, Buckinghamshire, UK). Images were scanned using a 2000 dpi slide scanner, mounted in Adobe (Mountain View, CA) Photoshop and printed on a dye sublimation printer. No signal was seen when the corresponding sense RNA probe was used as control. Additional hybridization with antisense RNA probes corresponding to hp 17–225 of the cDNA showed identical pattern of hybridization to that observed with bp 226–411.

Immunohistochemistry. To facilitate cellular staining with the CART antibody, deeply anesthetized (Avertin, Merck, Darstadt, Germany; 50 mg/kg) animals were injected with 100 μg of colchicine (Sigma, St. Louis, MO) in 10 ml of PBS into the lateral cerebral ventricle. Twenty-four hours later animals were reanesthetized and perfused transcardially, first with heparinized (15000 IU/l) KPBS, followed by 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffer (37°C), pH 7.4. The brains were removed and post-fixed with 4% PFA for four hours. Animals were reanesthetized and perfused transcardially with 100 ml of PBS containing 0.05% Tween 20, the sections were incubated at room temperature for 1 hr in a mixture of biotinylated swine anti-rabbit (1:500; Dako, Glostrup, Denmark) and Texas Red-conjugated sheep anti-mouse (1:50; Amersham). After three rinses in PBS with Tween 20 the sections were finally incubated for 60 min at room temperature in FITC-conjugated avidin and subsequently mounted in Glycergel and examined in a Zeiss (Thornwood, NY) LSM 510 confocal microscope.

Approximate percentages of co-localization (expressed as the percentage of a given cell population that was found to contain CART) were evaluated in images acquired from the confocal microscope and are given in Table 1.

Image editing software (Adobe Photoshop and Adobe Illustrator) was used to combine acquired images into plates, and figures were printed on a Tektronix (Wilsonville, OR) dye sublimation printer.

RESULTS

CART in situ hybridization

Figure 1 shows the distribution of CART mRNA in the hypothalamus of a non-treated rat (Fig. 1a,c,e,g,i,k) juxtaposed to photomicrographs of CART-IR (of approximate same level) in a colchicine-treated rat (Fig. 1b,d,f,h,j,l). The pattern of CART mRNA is similar to that reported by Douglass et al. (1995). The exact location of the cells expressing CART mRNA was determined from the emulsion-dipped, counterstained sections. The most rostrally located group of cells found to express CART mRNA was located in the periventricular nucleus (PeV) and extended from the rostral level of the suprachiasmatic nucleus to the level of the rostral tip of the ventromedial hypothalamic nucleus. Magnocellular neurons in both the supraoptic nucleus (SON) and the PVN were found to contain CART mRNA, although the signal here was rather low (Fig. 1a,c). The strongest signal in the PVN, however, was observed in the ventral part of the medial paraventricular subnucleus (Fig. 1c). Intense labeling was observed in the retrochiasmatic area (Fig. 1c), immediately rostral to the arcuate nucleus (Arc), which was found to express CART mRNA abundantly throughout its rostrocaudal extent (Fig. 1e,g,i,l). A high number of intensely labeled cells were found in the zona incerta (ZI), starting at the caudal end of the PVN (at the level of the lateral paraventricular subnucleus; Fig. 1e). In the caudal direction the ZI group of cells gradually extended laterally and ventrally into the lateral hypothalamic area (LHA), which contains the highest number of CART-expressing cells in the hypothalamus (Fig. 1g,l). The lateral hypothalamic group of cells was concentrated in the perifornical area (Fig. 1g, asterisk indicates location of fornix). The most caudal group of CART-expressing cells in the hypothalamus was detected in the ventral premammillary nucleus (Fig. 1k).

CART immunohistochemistry

Although the monoclonal antibody used to detect CART peptide-containing cells did stain neuronal-like cells in non-colchicine-treated material, cellular staining was greatly facilitated by colchicine treatment. As seen in Figure 1, b, d, f, h, j, and l, the distribution of CART-IR cells in colchicine-treated material exactly overlapped that described for the in situ hybridization, suggesting that all cells constitutively expressing CART are visualized. Colchicine treatment also facilitated cellular staining for the other neuropeptides and enzymes, greatly improving the results obtained in co-localization studies.
Double immunohistochemistry for CART and other hypothalamic neuropeptides

Figure 2 shows the extensive co-localization that was found of CART and POMC in the Arc (Fig. 2a) and CART and MCH in the ZI and LHA (Fig. 2b,c). In the Arc, almost all CART cells were found to contain POMC and vice versa (Fig. 2a) and this high degree of co-localization was evident throughout the rostro-caudal extent of the arcuate nucleus (data not shown).

In the LHA and ZI, CART immunoreactivity co-existed with MCH (Fig. 2b,c). In the rostral part of the ZI and the most medial part of the LHA these peptides were found to be co-stored in nearly every cell (Fig. 2b). In the more lateral and caudal parts of the LHA (perifornical nucleus and area medial to the internal capsule), an increasing number of MCH cells that were not immunoreactive to CART could be observed (Fig. 2c).

In the LHA and ZI the population of CART-IR cells was

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**Figure 1.** Distribution of CART mRNA and CART-IR in hypothalamus. Expression of CART mRNA as revealed by *in situ* hybridization (a, c, e, g, i, k) is juxtaposed to sections (approximately the same levels) immunostained for CART-IR with the monoclonal antibody used for the co-localization studies (b, d, f, h, j, l). Sections are organized from rostral (a) to caudal (l). Dark areas in a, c, e, and g indicate CART mRNA expression. In some areas individual cells stand out as intense black dots (notably in the ZI and LH). The asterisk in g indicates location of the fornix. Note that the *in situ*-hybridized sections are from a nontreated animal and 14 μm in thickness, whereas the immunostained sections come from a colchicine-treated animal and are 40 μm thick. *Arc*, Arcuate nucleus; *LH*, lateral hypothalamic area; *PeV*, periventricular nucleus; *PMV*, ventral premammillary nucleus; *PVN*, paraventricular nucleus of the hypothalamus; *RCh*, retrochiasmatic area; *SON*, supraoptic nucleus; *ZI*, zona incerta.
found to be completely segregated from the group of orexin B-containing cells in this area (Fig. 3b).

In the Arc no co-existence of CART with NPY or with TH was observed. The bulk of both NPY-immunoreactive (Fig. 3a) and TH-immunoreactive cells are located more medially in the Arc than the CART-containing neurons.

Both magnocellular and parvicellular subnuclei of the PVN were found to contain CART-IR neurons. In the magnocellular parts of the PVN (both anterior and posterior subdivisions) CART-IR was found to the largest extent in oxytocinergic neurons (Fig. 3d) and more rarely in the vasopressinergic neurons (data not shown). The same proportional distribution was found in the SON. Figure 3e shows co-localization between CART and vasopressin in the SON. In the parvocellular PVN, the most rostral group of CART-IR cells was found in the anterior subnucleus. Double staining for CART and GAL in this area showed that a few CART neurons also contained GAL-IR (Fig. 3f, arrows). Further caudally, at the level of the central portion of the PVN, two apparent populations of parvicellular neurons exist in the PVN, a medial periventricular co-localizing somatostatin and one in the ventral portion of the medial parvicellular subnucleus of the PVN (ventral part). Throughout the rostrocaudal extent of the PeV approximately half of the somatostatinergic neurons co-localized CART-IR (Fig. 3c). No co-localization between CART- and TH-positive neurons in the PeV was observed. In the medial parvicellular PVN, where the majority of hypofysiotrophic CRH neurons are located, double labeling revealed that CRH and CART neurons constitute two separate populations (data not shown).

In the mammillary region, where a small population of large CART neurons were found, double immunohistochemistry revealed that no CART-IR elements contained histamine (revealed with antibody to HDC; data not shown).

A summary of the distribution of co-localized cells is given in Table 1.

**DISCUSSION**

Using *in situ* hybridization and immunohistochemistry techniques, we have confirmed and extended previous observations on the distribution of CART mRNA and CART-IR in the rat hypothalamus. The distribution of CART-IR neurons within the hypothalamus as revealed using a monoclonal antibody raised against CART(41–89) overlapped exactly the pattern of CART mRNA, suggesting that the antibody is specific to CART and that the colchicine treatment used to enhance perikaryal staining did not induce CART expression in cells not normally expressing this peptide. The monoclonal antibody has been used to purify CART peptide from hypothalamic tissue and recognizes at least two forms of hypothalamic CART (Thim et al. 1999). CART(42–89) immunoreactive only for CART (red) is seen in the medial part of the Arc immediately lateral to the third ventricle (straight arrow). A few POMC cells not co-storing CART are also seen (green; curved arrow). A dense plexus of CART-only fibers are observed in the external layer of the median eminence, presumably arising from periventricularly located CART neurons (a, bottom left). b, In the ZI and rostral part of the LHA, all MCH cells are immunoreactive for CART (b, yellow). A number of cells located in the periventricular nucleus containing only CART are seen in the bottom left of b. c, In the caudal and lateral part of the LHA an increasing number of MCH cells are found that do not co-localize with CART (green). The vast majority of CART cells here also contain MCH (yellow). Scale bars, 50 μm.
has previously been isolated in ovine hypothalamic extracts, and this fragment corresponds to that predicted from possible sites of posttranslational processing of the mature CART(1–89) peptide (Thim et al., 1998).

One major finding is that CART is present in both classic neuroendocrine neurons and in hypothalamic projection neurons. Given the involvement of both the arcuate nucleus and the lateral hypothalamic area in feeding behavior, it is of particular interest that an endogenous anorectic peptide is highly co-localized with POMC in the Arc and MCH in the LHA and ZI. Central administration of CART(42–89) is anorectic in rats and induces c-fos expression in areas involved in feeding behavior (Kristensen et al., 1998; Vrang et al., 1998). Also, CART expression in arcuate neurons correlates intimately with leptin signaling with decreasing levels during fasting and in ob/ob mice being reversed by treatment with exogenous leptin (Kristensen et al., 1998).

The presence of extensive co-storage within the Arc of CART and POMC is interesting because these cells contain the signaling form of the leptin receptor (Cheung et al., 1997), implying that the effects of leptin on CART and POMC expression are direct (Schwartz et al., 1997; Mizuno et al., 1998). In the Arc POMC is processed to yield β-endorphin and α-melanocyte-stimulating hormone (α-MSH). α-MSH potently inhibits food intake when administered intracerebroventricularly (Fan et al., 1997), an effect that is believed to be mediated by hypothalamic melanocortin 3 and 4 (MC3 and MC4) receptors, because antagonists of these block α-MSH induced anorexia and stimulates food intake in free-feeding animals (Fan et al., 1997; Huszar et al., 1997).

Arcuate POMC neurons project to the medial parvocellular subnucleus of the PVN where released peptides exert effects on both feeding behavior and hypophysiotrophic CRH neurons (Guy et al., 1981; Pickut, 1985; Baker and Herkenham, 1995). However, the predominant input of melanocortinergic and β-endorphinergic fibers to the PVN makes synapses on neurons in the ventral portion of the medial parvocellular subnucleus, giving rise to long, descending projections to the lower brainstem and intermediolateral column of the spinal cord (Kiss et al., 1984; Pickut, 1985). In addition to anorectic actions, central administration of the MC3 and MC4 agonist MTH also increases sympathetic drive in mice (Fan et al., 1998), and direct administration of melano-

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**Figure 3.** CART co-localization with other hypothalamic neurotransmitters. Confocal laser scanning images show dual-labeling pattern of CART immunoreactivity together with immunoreactivities for NPY (a), orexin B (b), somatostatin (c), oxytocin (d), vasopressin (e), or galanin (f). a, In the arcuate nucleus CART-IR neurons (red) are larger and distributed more laterally than NPY neurons (green). No co-localization is seen between these two peptides. b, In the lateral hypothalamic area it is evident that CART and orexin B constitute two nonoverlapping populations of neurons. c, Scanning image from the central part of the PVN showing co-localization between CART and somatostatin (yellow neurons). It is seen that an additional population of CART-IR cells (red) are found in the ventral part of the medial parvicellular PVN. The third ventricle is located in the left of c. d, Double staining for CART (red) and oxytocin (green) showing co-localization in both magnocellular as well as parvocellular neurons (yellow). e, Co-localization between CART and vasopressin in the supraoptic nucleus. f, In the anterior parvocellular PVN, few galaninergic neurons were found to contain CART (arrows point to double-stained cells). However, the majority of CART-containing (red) and galanin-containing (green) cells were segregated. Scale bars, 50 μm.
cortin agonist into the PVN increases energy expenditure (R. D. Cone, personal communication). Thus it is possible that CART in concert with α-MSH influences the tone of sympathetic outflow via the PVN. Our finding of a high degree of co-storage of CART and POMC in the Arc, the anorectic properties of both peptides, and the inducibility of CART and POMC in the Arc by leptin strongly suggests that these peptides act in concert to downregulate food intake.

The complete segregation of NPY and CART within the Arc fits well with the other data from the present study showing almost 100% co-localization between CART and POMC, as other studies have shown that NPY and POMC (α-MSH) indeed constitute two different populations of neurons within the Arc (Chronwall, 1985). Recently, an endogenous antagonist of the melanocortin 3 and 4 receptor antagonist has been described (Fong et al., 1997; Ollmann et al., 1997; Shutter et al., 1997). This peptide, termed agouti-related protein (AgRP), co-exists with NPY in Arc neurons (Broberger et al., 1998), and a stimulatory role of AgRP on feeding behavior is suggested by experiments showing increased AgRP expression in ob/ob mice and obesity in transgenic animals expressing AgRP ubiquitously (Ollmann et al., 1997). Also, C-terminal fragments of AgRP potently stimulate food intake when injected intracerebroventricularly (Rossi et al., 1998).

Table 1. Immunohistochemical characterization of CART neurons

<table>
<thead>
<tr>
<th>Neuron</th>
<th>Approximate co-localization (%)</th>
</tr>
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<tbody>
<tr>
<td>POMC</td>
<td>&gt;95</td>
</tr>
<tr>
<td>MCH</td>
<td>&gt;95</td>
</tr>
<tr>
<td>MCH</td>
<td>54</td>
</tr>
<tr>
<td>SOMA</td>
<td>38</td>
</tr>
<tr>
<td>OXY</td>
<td>31 (PVN)</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>15 (PVN)</td>
</tr>
<tr>
<td>GAL</td>
<td>11</td>
</tr>
<tr>
<td>Orexin B</td>
<td>LH</td>
</tr>
<tr>
<td>NPY</td>
<td>Arc</td>
</tr>
<tr>
<td>CRH</td>
<td>Medial parvicellular PVN</td>
</tr>
<tr>
<td>TH</td>
<td>PeV, Arc, and ZI</td>
</tr>
<tr>
<td>HDC</td>
<td>Mammillary region</td>
</tr>
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</table>

From our data and others, it is therefore evident that the Arc houses at least two populations of neurons with opposite effect on food intake and energy balance, one consisting of NPY-AgRP neurons with feeding-stimulatory effects and the other consisting of POMC-CART neurons with negative effects on energy balance.

The other major population of CART neurons in the hypothalamus that is interesting in terms of regulation of food intake is the population found within the ZI and LHA. The distribution of MCH-IR cells found in the present study completely overlaps that described previously (Skofitsch et al., 1985; Bittencourt et al., 1992). An almost total overlap between CART- and MCH-IR elements was observed in the rostral ZI and medial and rostral parts of the LHA, whereas in more caudal and lateral parts of the LHA an increasing number of MCH-IR cells was found not to contain CART. A role for MCH in regulation of feeding behavior has recently been proposed, because MCH mRNA in the LHA is increased in ob/ob mice (Qu et al., 1996), and MCH injected intracerebroventricularly stimulates food intake in the rat (Qu et al., 1996; Rossi et al., 1997; Ludwig et al., 1998). In light of these data, it is possible that the function of CART within the melanocyte-stimulating hormone cells is to counteract the effect of MCH when, presumably, co-released with this orexigenic peptide. The MCH knock-out mouse is hypophagic and displays a leaner than normal phenotype, suggesting a shift toward anorexia, which may be explained by increased CART tone of the LHA neurons normally expressing MCH (Shimada et al., 1998). Future studies of CART expression in this mouse model are of great interest. A completely different role of CART within this system, however, cannot be excluded.

Interestingly, another orexigenic peptide present in neurons of the LHA, orexin B, was never co-localized with CART. Orexin B (hypocretin B) is one of two peptides (A and B) cleaved from the same precursor and confined to neurons in the LHA (de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). Evidence in support for a stimulatory role in feeding is given by the fact that orexin mRNA is increased with fasting, and orexin peptide elicits feeding when injected intracerebroventricularly (Sakurai et al., 1998). Our results thus suggest that CART-MCH and orexin B cells constitute two separate populations of cells, which is in agreement with a recent study demonstrating no overlap of hypocretin B and MCH immunoreactivities in rat LHA (Peyron et al., 1998). Further studies are needed to clarify whether orexin-containing cells and MCH- and CART-containing cells project to the same target or have divergent targets.

In the PVN, CART-immunoreactive neurons were observed in areas known to harbor neuroendocrine cells as well as in subnuclei containing neurons projecting to preganglionic autonomic cells of brainstem and spinal cord. The parvocellular neurons of the periventricular strata are mainly hypophysiotrophic and project to the median eminence (Larsen et al., 1991; Merchenthaler, 1991). Given the anatomical localization and co-existence with somatostatin, it is evident that CART-IR parvocellular neurons in the PeV and PVN are neuroendocrine cells possibly contributing to the dense innervation of the portal capillaries in the external zone of the median eminence (Koylu et al., 1997). The functional implications of this co-existence are speculative, but a role for CART as a hypophysiotrophic modulatory transmitter seems plausible. Other input to the external zone of the median eminence may arise from galanin-containing neurons co-localizing CART in the anterior parvocellular PVN. The higher levels of galanin expression in this part of the PVN in obesity-prone animals and the positive correlation between hypothalamic galanin expression and dietary fat suggest that CART co-existing in these neurons could somehow modulate the galanin orexigenic potential (Leibowitz et al., 1998).

The majority of CART-IR in magnocellular neurons in the PVN and SON was oxytocinergic, suggesting that CART could influence neurohypophysial neuropeptide release. The addition of yet another peptide to the long list of neurotransmitters co-expressed in magnocellular hypothalamo-neurohypophysial neurons further emphasizes the impressive expression potential of
these neurons (Meister et al., 1990). Some of the oxytocin neu-
rons co-localizing CART were parvicellular and confined to the
ventral portion of the medial paraventricular subnucleus. This re-
region sends long, descending projections to autonomic preagnosti-
onic cells, emphasizing that CART may act in concert with oxytocin, vasopressin, and Met-enkephalin on these cells (Cechetto and Saper, 1988).

In conclusion, we have shown that CART is present in numer-
ous hypothalamic cell groups affecting feeding behavior. How-
ever, it is not possible from the content of CART to assign
stimulatory or inhibitory effects on feeding for a specific neuron.
Also, neuroendocrine systems may have their final output influ-
enced by CART co-existing with classic hypothalamic factors as
well as neurohypophysial hormones.

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