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Research report

CART peptide diurnal rhythm in brain and effect of fasting

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Abstract

We have recently shown that CART peptides exhibit a diurnal rhythm in blood that is affected by food intake and glucocorticoids. In the present study, we extend our observations by demonstrating that CART peptides also exhibit a diurnal rhythm in several brain regions, notably the nucleus accumbens, hypothalamus and amygdala, but not in the midbrain. To examine whether the CART peptide rhythm was dependent on food intake, animals were food-deprived for 24 h. In regular-fed animals, CART peptide levels were lower in the morning compared to evening hours. However, this diurnal variation of CART peptide was not apparent in fasted animals, and CART peptide levels were reduced. The diurnal variation of CART mRNA in the nucleus accumbens paralleled the variation of CART peptide in this region. Similar to the peptide, the mRNA did not change in midbrain. These results show that CART peptide levels and gene expression undergo a diurnal variation in some brain regions, and the variation is altered by fasting. These findings suggest a variety of regulatory mechanisms for CART and additional considerations for CART's role in brain.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Other neurotransmitters

Keywords: Feeding; Drug abuse; Cocaine; Amphetamine-regulated transcript

1. Introduction

CART peptides are neurotransmitters involved in a variety of processes, including the action of psychostimulants [7,12], feeding and body weight [11,14,16,17,25], stress and glucocorticoids [1,14,26], endocrine control [13,18,24,27] and other processes [11]. Recent studies have demonstrated the presence of CART peptides in blood and that they undergo a diurnal variation that may be related to glucocorticoids [21,22]. Related to this, the CART promoter has been shown to have an E-box transcription factor binding site [6] that may play a role in circadian rhythmicity [10]. In view of this previous evidence, we speculated that CART peptide may exhibit a diurnal rhythm in the brain. To test this important possibility, we have examined CART peptide and mRNA in several brain regions at various times. These regions include the hypothalamus, the nucleus accumbens, amygdala and the midbrain. In addition to the role of CART in feeding that is linked to its distribution in the hypothalamus, the presence of CART in the nucleus accumbens and amygdala implicates this peptide in rewarding behavior. Thus, the potential existence of the diurnal rhythm of CART in these areas could suggest that its diurnal periodicity plays a role in feeding and reward. Because fasting-related changes in CART gene expression have been previously shown [4,16,23] and because fasting was shown to influence the rhythm of blood CART levels [22], we also measured the effects of food deprivation on CART levels and its diurnal variation in brain.

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2. Methods

2.1. Animals and procedures

Male Sprague–Dawley rats (350–375 g, Harlan, Indianapolis, IN) were housed in groups of two and maintained on a 12-h normal light/dark cycle with lights on at 6 a.m. The ambient temperature was maintained between 20 and 25 °C. Water and food (rodent diet 5001, Lab Diet, Minneapolis, MN) were provided ad libitum. Animals were sacrificed by decapitation at varying times, and several brain regions were dissected and assayed for CART peptide levels by radioimmunoassay (RIA). For determining the effects of fasting on CART expression levels and the diurnal rhythm, animals were also fasted for 24 h before sacrifice. All procedures were carried out in accordance with an Emory-IACUC-approved protocol.

2.2. Radioimmunoassay

The concentration of immunoreactive CART peptide was determined by radioimmunoassay (RIA). The procedure was carried out generally as previously described [19] but with a commercially available ^{125}I-RIA kit provided by Phoenix Pharmaceuticals (Belmont, MA), [22]. RIA kit was validated before use with tissue extracts and dose–response curves with increasing concentration of authentic CART 55–102 standard (Peptide International, Louisville, Kentucky). CART 55–102 is a naturally occurring active fragment that is commercially available [11]. The assay sensitivity was 10 pg/tube, and intraassay variability was 5%. Samples were purified by Sep Pak C18-E columns (Phoenix Pharmaceuticals). The eluates were freeze-dried overnight using a lyophilizer, and samples were dissolved in RIA buffer. Added internal standards (80 pg/tube) of authentic CART 55–102 were recovered to a level of 57%, and the duplicate values deviated less than 5%. All final values are corrected to 100% recovery.

Extraction of CART peptide from tissues was carried as previously described by Murphy et al. [19]. Briefly, 0.5 ml of 0.1 M acetic acid was added to each tissue sample, which was then transferred to boiling water bath for 15 min. After cooling on ice, samples were homogenized and centrifuged at 13,000 × *g*/15 min. The supernatant was collected, and the pellet was reextracted with acetic acid. The supernatant was divided into two tubes. One tube was used for RIA analysis of CART peptides and the other for quantification of soluble protein. The supernatants were pooled and further purified on Sep Pak C18-E columns. The eluates were freeze-dried overnight using a lyophilizer, and samples were dissolved in RIA buffer before assay.

2.3. Real-time PCR for CART mRNA

Midbrain and nucleus accumbens CART mRNA levels were quantified by real-time PCR. 18s Ribosomal RNA was used as an endogenous control to correct for potential variation in RNA loading. The primers and Taqman probe for CART were designed using the Primer Express software (Perkin-Elmer Applied Biosystem) based on sequence from the Genbank database (forward primer: CAAGAGTAAACGCATTCCGATCT; reverse primer: CACTGCTCTCCAGCGTCACA; Taqman probes: TGAGAAGAAGTACGGCCAAGTCCCCA). The primers and Taqman probe for 18s RNA were purchased from Applied Biosystem. Total cellular RNA was extracted using TRIzol Reagent (Gibco BRL, Invitrogen) according to the manufacturer's instructions. The RNA ratio was determined spectrophotometically, with a ratio of optical density 260:280 exceeding 1.6 considered relatively pure. Real-time PCR was performed using a 7700 Detection System (Applied Biosystem). The reaction was carried out in a 25 μl reaction mixture containing 1 × Taqman Buffer A, 5.5 mM $CaCl_2$, 300 μM dATP/dCTP/dGTP, 600 μM dUT, 0.25 U/μl MultiScribe Reverse Transcriptase, 0.025 U/μl AmpliTaq Gold DNA Polymerase, 0.4 U/μl RNase Inhibitor, 400 nM primers, 250 nM Taqman probe and 60 ng total RNA per sample. Reactions in which RNA was omitted served as negative controls. Thermal cycling conditions included the following steps: an initial 30 min at 48 °C for reverse transcription, a preheating for 10 min at 95 °C to activate Taqman polymerase followed by 40 cycles of two steps of PCR consisting of 15 s at 95 °C and 1 min at 60 °C. Samples were amplified simultaneously in quadruplet in each assay. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye from Taqman probe. A standard curve was used for relative quantitation.

3. Results

As Fig. 1 shows, CART peptide levels in the nucleus accumbens exhibited a daily rhythm with values that were

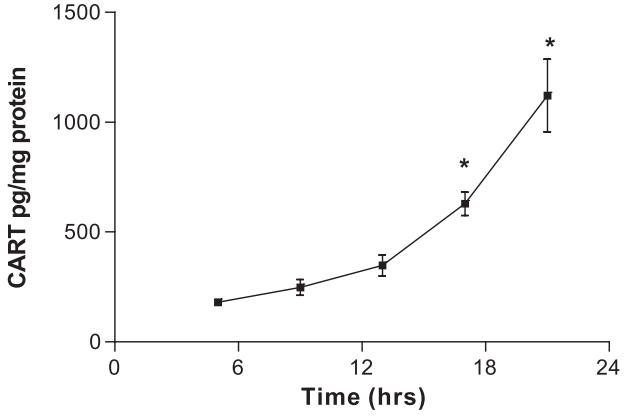

Fig. 1. The diurnal profile of CART peptide in nucleus accumbens. The data represent the mean ± S.E.M. obtained from six to eight rats per time. Data were analyzed by one-way ANOVA: ($F_{(4,34)}$ = 26.7 $p < 0.0001$) followed by a Newman–Keul's post hoc test. * represents levels of CART peptide at 17:00 and 21:00 h that were significantly different ($p < 0.0001$) from those obtained 05:00, 09:00 and 13:00 h.