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## Wake promoting effects of cocaine-and amphetamine-regulated transcript (CART)

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### Abstract

Cocaine- and amphetamine-regulated transcript (CART) peptides modulate anxiety, food intake, endocrine function, and mesolimbic dopamine related reward and reinforcement. Each of these disparate behaviors takes place during the state of wakefulness. Here, we identify a potential wake promoting role of CART by characterizing its effects upon sleep/wake architecture in rats. Dose-dependent increases in wake were documented following intracerebroventricular CART 55–102 administered at the beginning of the rat's major sleep period. Sustained wake was observed for up to 4 hours following delivery of 2.0 µg of CART peptide. The wake promoting effect was specific to active CART 55–102 because no effect on sleep/wake was observed with the inactive form of the peptide. Increased wake was followed by robust rebound in NREM and REM sleep that extended well into the subsequent lights-off, or typical wake period, of the rat. These findings point to a potential novel role for CART in regulating wakefulness.

### Keywords

hypothalamus; rebound; hypocretin; sleep

### Introduction

The timing and maintenance of wakefulness requires coordination of disparate homeostatic functions, *e.g.*, endocrine, metabolic, autonomic, and behavioral arousal that are critical to an organism's survival (Adamantidis and de Lecea, 2008). Key mediators of alertness include hypothalamic peptides such as hypocretins whose loss causes the prototypical disorder of daytime somnolence, *viz.*, narcolepsy/cataplexy (Nishino, 2007). Cocaine and amphetamine regulated transcript (CART), an abundant hypothalamic neuropeptide, is an attractive additional candidate for modulates alertness. It is involved in feeding, energy homeostasis, and modulating the HPA axis (Kuhar et al., 2000) and its localization outside of the hypothalamus—for example in neurons of the nucleus accumbens, synaptic terminals of the ventral tegmental

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area and substantia nigra, in part engaging mesolimbic dopamine circuits, suggests additional influences upon reward/motivation and locomotion (Smith et al., 1999, Dallvechia-Adams et al., 2002, Rogge et al., 2008). Many of these same brain pathways are intimately involved in promoting wakefulness (Rye, 2004). This lead to our central hypothesis that CART also participates in homeostatic mechanisms integral in governing the waking state.

The wake promoting psychostimulants cocaine or amphetamine transcriptionally upregulate CART mRNA in the ventral striatum (Douglass et al., 1995). Since this discovery, CART has been identified as a brain-gut peptide neurotransmitter affecting a wide array of physiological functions (see August 2006 issue of *Peptides* for reviews). A role in the modulation of thalamocortical arousal state (i.e., wake vs. sleep) is suggested by the localization of CART in the brainstem reticular regions and cholinergic nuclei near the pontomesencephalic junction, the locus coeruleus, and the raphe nuclei (Koylu et al., 1998; 1999). In addition, CART is expressed in neurons and synaptic terminals in the lateral hypothalamic area (LHA)/perifornical region where it colocalizes in a species specific manner with somnogenic melanin-concentrating hormone (MCH) and the wake promoting peptide hypocretin (Broberger et al., 1999, Elias et al., 2001, Koylu et al., 1998, Peyron, 2006, Hanriot et al., 2007). Moreover, diurnal rhythms of CART peptides are present in brain and periphery (Vicentic, 2006). It remains unknown if CART modulates sleep-wake states. To address this void in knowledge, we examined the effect of intracerebroventricular (icv) administration of CART 55–102 in rats upon sleep/wake architecture.

## Methods

### Subjects

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Two groups of male Sprague-Dawley rats (Charles River; Group 1: n = 12; Group 2: n = 10) weighing 350–400 grams were used. Throughout the protocol, rats were housed under a 12:12 hour light:dark cycle (lights on at 7am) with food and water provided ad-libitum.

### EEG Surgery

To record electroencephalographic (EEG) activity, animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and placed into a stereotaxic frame. Four holes were drilled in the skull and two pairs of sterile 0–80 × 3/32 screw electrodes (Plastics One, Roanoke VA) inserted. With reference to bregma, the first electrode pair was placed AP –1.5 mm, ML 3.0 mm and AP –6.3 mm, ML 3.5 mm. These lateral coordinates facilitate recording of cortical alpha and delta wave activity. The second electrode pair was placed contralaterally at AP +2.5 mm, ML 1.5 mm and AP –3.6 mm, ML 1.5 mm. These medial coordinates enable recording of theta wave activity. For recording of neck EMG activity, one fine wire (40 gauge Cooner Wire, Chatsworth, CA), was inserted into the left nuchal muscle while a second fine wire was inserted into the right nuchal muscle. After placement, EEG and EMG electrodes were attached to a micro-connector (Continental Connector Corp) and a dental acrylic compound (Plastics One, Roanoke VA) poured over the screws, wires, and connector to ensure fixation of the assembly to the skull. Once the acrylic skullcap dried, the free-ends of the incision were sutured, thereby leaving the micro-connector pins exposed and unencumbered.

### Intracerebroventricular (ICV) cannula placement

Following placement of EEG screw electrodes, a fifth hole was drilled in the skull overlying the lateral ventricle. With reference to bregma, co-ordinates for guide cannulae (Plastics One, Roanoke, VA) placement are: AP: –1.0 mm, ML + 1.5 mm, DV – 2.8 mm. Guide cannulae were secured with dental acrylic at the same time the EEG screws and micro-connector were

covered with acrylic. A dummy cannula was then inserted into the guide cannula. After a 5–7 day recovery, the accuracy of the guide cannula location was confirmed using an angiotensin drink response test. First, the rat was removed from its home cage and the dummy cannula replaced by an internal cannula (with 1 mm projection past the end of the guide cannula) attached to PE 50 tubing (Plastics One, Roanoke, VA), and 4  $\mu$ l of 0.15 M saline (i.e., control) injected using a 10  $\mu$ l Hamilton syringe. Upon return to its home cage the amount of water consumed over the subsequent 30 minutes was recorded. After approximately 1 hour, this process was repeated employing 100 ng of angiotensin II (Sigma, St. Louis, MO) in 4  $\mu$ l of 0.15 M saline. Only rats whose water consumption following angiotensin exceeded saline consumption by at least 4mls were employed in the full experimental protocol. Three rats failed to consume the minimum amount of water and were not included in any further testing or analysis.

### Sleep-Wake recording

Following recovery from surgery, animals were placed into a sound attenuated, ventilated, and light-regulated environmental cubicle (BRS/LVE Davis, Maryland) and EEG/EMG wires were attached via a cable tethered to a counterbalanced and suspended commutator. The cubicle's internal dimensions were 35" high, 34" wide and 29" deep. A heavy gauge metal dividing wall separated the inside of the cubicle into two individual subunits. Each subunit within the cubicle is self-contained with individual air inlet ports, exhaust fans, and adjustable light source. The recording cubicles were maintained on a 12:12 hr light-dark cycle (lights-on at 7 am), food and water was provided *ad libitum* throughout and the ambient temperature maintained between 76–80 degrees F.

**Data collection - EEG and EMG**—After two days of acclimatization to the cubicle and the EEG recording tether, continuous, uninterrupted acquisition of electrophysiological measures of sleep-wake behavior ensued. The EEG, theta, and EMG signals were preamplified by Grass model 12A5 amplifiers; EEG and hippocampal theta signals were low-pass filtered at 0.3Hz and high-pass filtered at 30Hz while EMG signals were low-pass filtered at 10Hz and high-pass filtered at 100Hz. The amplified and filtered signals were outputted to a National Instruments analog-to-digital converter (PCI-MIO-16E-4), digitally processed, and viewed on a real-time basis via Somnologica Science®, Medcare, Reykjavik, Iceland). At the termination of the recording period, all data was stored on CD diskettes at 400Hz resolution for subsequent offline scoring and analysis.

### Experimental protocol

Following acclimatization to sleep-recording cubicle, baseline data was recorded for 24–48 hr. Group 1: On subsequent days rats received randomized icv delivery of either 0.15 M saline vehicle, 0.5, 1.0 or 2.0  $\mu$ g CART 55–102 (Santa Cruz Biotechnology Inc., Santa Cruz, CA)/ 2  $\mu$ l circadian time (CT) 0–1 (i.e. 7–8am; lights-on at 7 am). Group 2: On subsequent days rats received randomized icv delivery of either 0.15 M saline vehicle, or inactive CART peptide, CART 1–27 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), at doses equivalent in molarity to delivery of either 2.0 or 5.0  $\mu$ g CART 55–102/2  $\mu$ l at CT 0–1. The following 23 hours of sleep/wake were recorded as post-treatment data. At the end of experiments, rats were terminated and brains histologically processed to verify location of cannula placement.

### Perfusion/fixation and histochemistry

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4) and brains were processed histologically according to standard procedures to verify cannula placements.

## Scoring of sleep-wake architecture and analysis

Sleep-wake architecture data was scored on an epoch-by-epoch basis by one scorer blind to the experimental condition. Each 10-second epoch was scored as either wakefulness (W), non-rapid eye movement sleep (NREM) or rapid-eye movement (REM) according to standard criteria. The total amount of time spent in each stage was calculated for each hour. All data was then entered into a SPSS (SPSS Inc., Chicago, IL) spreadsheet for statistical analysis. Data was analyzed using non-parametric statistics [Kruskal-Wallis (K-W) test].

## Results and observations

ICV administration of saline was followed by normal sleep/wake architecture and electroencephalographic (EEG) activity over the 24 hour recording period. There were no differences between Group 1 and Group 2 following 0.15 M saline administration. Administration of 0.5  $\mu\text{g}$  CART 55–102 did not significantly alter sleep/wake architecture. CART 55–102 increased the amount of time spent awake in the first 9 hours following peptide delivery, particularly for the 2.0  $\mu\text{g}$  dose (all effects significant for hours 1–4 at  $p < 0.0005$ , thereafter  $p < 0.05$ ) (see Figure 1a). Decreases in NREM sleep (Figure 1b) with higher dose were seen in the first 6 hours (all effects significant at  $p < 0.002$ ; hour 8 significant at  $p < 0.001$ ). REM sleep durations (Figure 1c) were not only reduced, but significantly suppressed for both higher active CART dosages relative to vehicle, inactive CART and active CART dosed at 0.5  $\mu\text{g}$  for the first 11 hours (all effects significant at  $p < 0.005$  for the first 9 hours;  $p < 0.01$  for hour 10 and  $p < 0.05$  for hour 11). The enhancement of wake observed following 2.0  $\mu\text{g}$  CART was dramatic with all subjects exhibiting long periods of consolidated (i.e., sustained) wakefulness (Figure 2).

Behavioral and electrographic evidence of epileptic activity as we have reported for 5.0  $\mu\text{g}$  CART doses (Keating et al., 2008), were not observed with the 0.5  $\mu\text{g}$ , 1.0  $\mu\text{g}$ , and 2.0  $\mu\text{g}$  doses employed here.

During the dark phase (during which rats are normally active), CART effects had largely disappeared when considered on an hour by hour basis (Figure 1a–c). However, when the 12 hours of the lights-off period were collapsed into a single time block, the analyses indicated that the 1.0  $\mu\text{g}$  and 2.0  $\mu\text{g}$  dosages of active CART were associated with less time awake ( $p < 0.001$ ), more NREM ( $p < 0.0005$ ), and more REM sleep ( $p < 0.001$ ), relative to 0.5  $\mu\text{g}$  active CART, inactive CART or vehicle control. This rebound sleep recovery response is represented in Figure 3.

## Discussion

Here we demonstrate a potential role for CART 55–102 (cocaine- and amphetamine-regulated transcript), a peptide concentrated in the hypothalamus, in promoting wakefulness. When injected intracerebroventricularly into rats, the active 55–102 versus inactive 1–27 recombinant peptides promoted wakefulness in a dose dependent fashion followed by compensatory rebound sleep, and occurred independently of any obvious enhancement of locomotion or electrographic or behavioral seizures. The effect of CART upon wakefulness was robust as evidenced by its emergence and sustained expression during a period of maximal sleep pressure, and a duration of wake promotion and intensity of sleep rebound matching or exceeding that reported for other wake-promoting agents (e.g., systemically delivered amphetamines or modafinil, and ICV administered hypocretin). Whereas 10–30  $\mu\text{g}$  hypocretin increases in wake become evident only 2–3 hours after icv delivery and do not extend beyond the 4<sup>th</sup> hour of recording (Hagan et al., 1999; Piper et al., 2000), 2  $\mu\text{g}$  CART enhancement of wake is evident within the first hour, and a greater than doubling of wake is still evident 6 hours post injection.

Our findings suggest that CART peptide activates cellular and subcellular signaling networks that produce long lasting changes in wake promoting brain circuits similar to the longevity of its effects on other behaviors commented upon previously (Vicentic et al., 2006). A comprehensive ascertainment of the cellular and subcellular substrates mediating the wake promoting effects of CART reported here is limited by methodology, and also by the ubiquitous localization of CART cell bodies and terminal fields in several brain areas known to promote wake or inhibit sleep. The 1.0–2.0ug doses of CART 55–102 administered intracerebroventricularly, albeit a log fold less than hypocretin doses necessary to elicit comparable wake promoting effects (Hagan et al., 1999; Piper et al., 2000), exceed the several hundred picograms/ml naturally present in mammalian cerebrospinal fluid (personal observations). The short latency (e.g., tens of minutes) to CART's wake promoting effects as opposed to the longer latencies observed with hypocretin [(e.g., hours) (Hagan et al., 1999; Piper et al., 2000)], suggest rapid engagement of wake promoting neural networks- or inhibition of sleep promoting neurons (e.g. the anterior preoptic area) in close proximity to circumventricular organs or ventricular system inclusive of the hypothalamus. HPA activation (Buckley and Schatzberg, 2005), the perifornical LHA, and hypothalamic hypocretin neurons (Mignot et al., 20002; Scammell, 2003) each promote arousal, and each are attractive candidates for mediating a hypothesized role for CART in promoting wakefulness. The extent of wakefulness that we observed may indicate a role of either direct or indirect stimulation on the arousal producing hypocretin system.

The hypothalamus contains the densest concentrations of CART cell bodies and terminals inclusive of the arcuate and paraventricular nuclei (PVN), and the dorsomedial nucleus and lateral hypothalamic/perifornical areas (Elias et al., 2001). Within the hypothalamus CART inhibits feeding, increases energy expenditure, and activates the HPA axis via the PVN and a corticotrophin-releasing factor receptor-dependent mechanism (Elias et al., 2001; Smith et al., 2004). CART is anorexigenic and curiously colocalizes in a species specific manner with two peptides known to be orexinogenic: one, MCH being somnogenic (Fort et al., 2009) and the other, hypocretin, being wake promoting. In rats, CART is expressed in ~80% of MCH containing neurons the majority of which are REM-sleep active (Hanriot et al., 2007) and a distinct subpopulation from adjacent hypocretin neurons (Broberger et al., 1999; Elias et al., 2001; Peyron, 2006). In humans, while the overlap of CART with MCH is retained, there is a significant coexpression of CART and hypocretin in individual neurons (Peyron, 2006; personal observations). Shared arousal-promoting roles for CART and hypocretin are evident not only in their close proximity or coexpression, but also in their common fields of synaptic termination in regions implicated in wake promotion such as the dopaminergic ventral tegmental area (VTA) and medial substantia nigra (SN), cholinergic magnocellular basal forebrain (MBF) neurons (Koylu et al., 1998; Smith et al., 1999; Elias et al., 2001; Dallvechia-Adams et al., 2002) and midline, paraventricular thalamus (Kirouac et al., 2006). While CART's physiological effects in the hypothalamus and other brain regions are evident in its calcium-dependent release from hypothalamic explants (Murphy et al., 2000) and unit recordings in hypothalamic slices (Davidowa et al., 2003), identification of CART specific receptors remains an important goal in order to attribute function to this compelling anatomy (Vicentic et al., 2006).

The long time course of exogenous CART's robust promotion of wake observed here reveal something of its potential mechanisms of action. The duration of effect is similar to that observed with traditional psychostimulants and modafinil, whereas the rebound sleep suggests actions beyond the dopaminergic synapse. The wake promoting profile and potency of 2 µg CART 55–102 is comparable to those seen following 1 mg/kg methamphetamine, 2.5 mg/kg d-amphetamine, 100–128 mg/kg modafinil and 20 mg/kg GBR-12909 (Edgar and Seidel, 1997; Wisor et al., 2001; Minzenberg and Carter, 2008). Also comparable to the amphetamines, CART administration produces robust rebound sleep, a compensatory response driven by sleep

homeostatic pressure (Minzenberg and Carter, 2008) and complex, nonspecific actions at monoamine transporters (Wisor et al., 2001; Minzenberg and Carter, 2008). The presence of rebound hypersomnolence following CART 55–102 then suggests actions beyond its known ability to increase synaptic dopamine availability (Yang et al., 2004; Shieh and Yang, 2008) such as potential engagement of additional monoaminergic, or even cholinergic, systems.

The results of the current study are not without certain limitations. The intracerebroventricular mode of delivery does not allow for specification of site of action. Ventricular diffusion is broad and so future work will concentrate on direct infusions on targeted nuclei to more precisely define structure-function relationships. Also, this study did not examine the effect of intrinsic CART on the microarchitecture of the electrographic signals used to characterize sleep-wake states. Future studies addressing the role of intrinsic CART will be multi-fold. CART knock-out mice, both homozygous and heterozygous will be critical to phenotype extensively in order to further elucidate CART's contributions to behavioral state control and the mechanisms underlying them. Separately, the infusion of antibodies against CART will be an important avenue of investigation into the role of endogenous CART upon state control. Finally, examination of the role of CART in the human condition would be a powerful tool to look at clinical correlations across disease state. Patients with problems across the sleep-wake continuum could be examined and the effect of their condition on CART as measured in their cerebrospinal fluid could provide a powerful window for further assessment of this peptide's role in state control.

On a final note, other explanations for the effects of CART on behavioral output need to be considered. While the overall behavioral readout here was an unequivocal increase in wake, there are potential confounds. CART is known to induce anxiogenic-like behaviors in rats and mice (for review see Stanek, 2006). The increased wakefulness seen here may therefore be an epiphenomenon of increased stress. The performance on behavioral measures of anxiety and locomotor activity was not performed but could be addressed by combining EEG monitoring with appropriate behavioral assessments to yield a more comprehensive picture of CART's effects on behaviors occurring on the background of wake.

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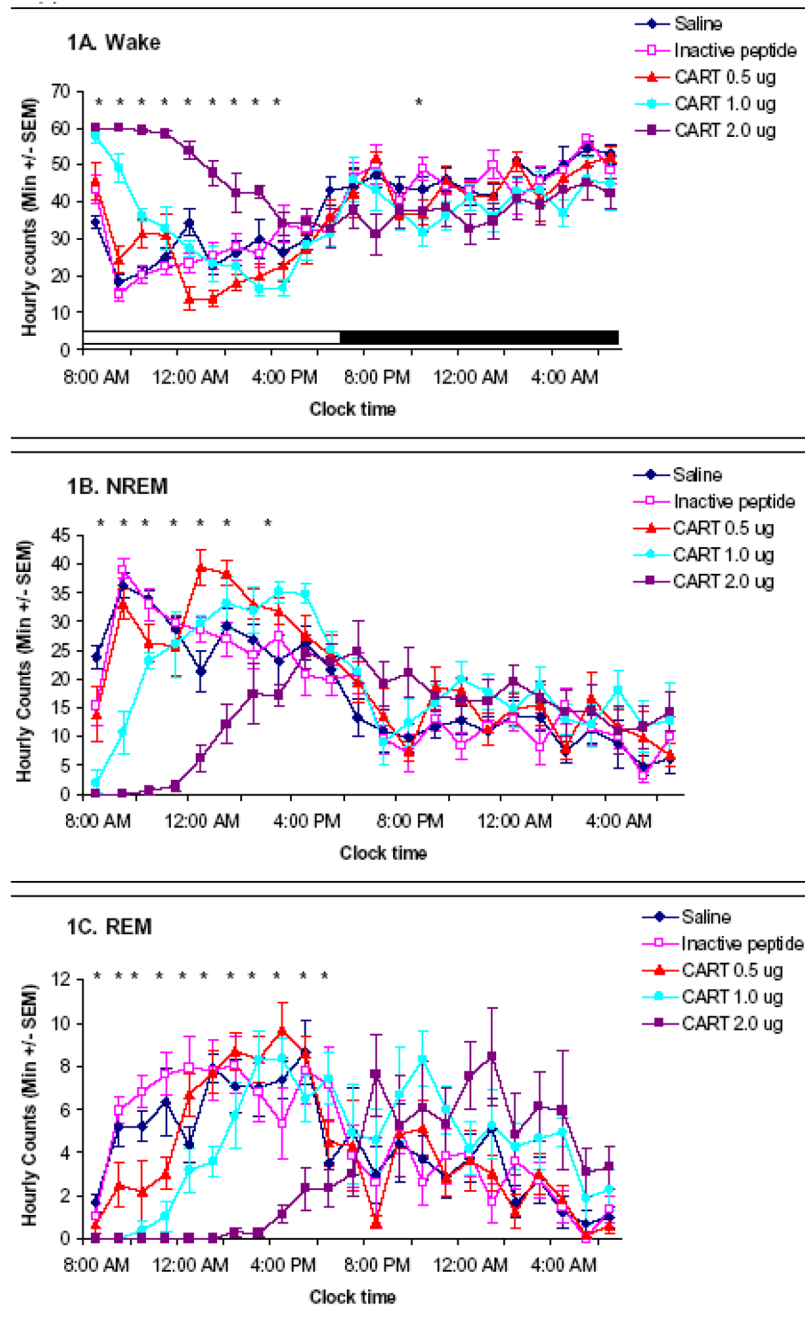
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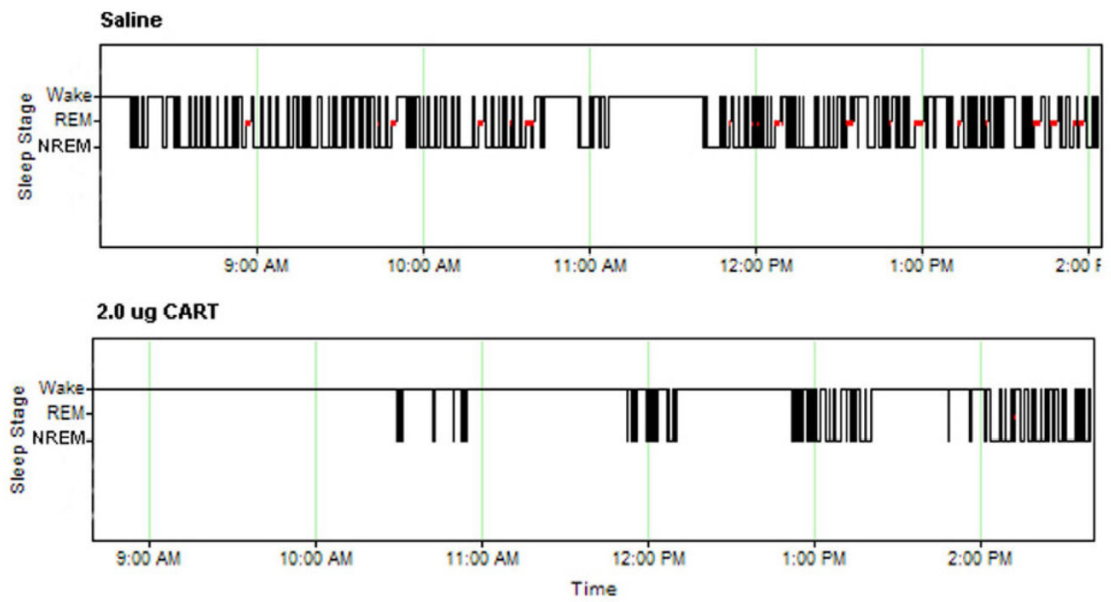
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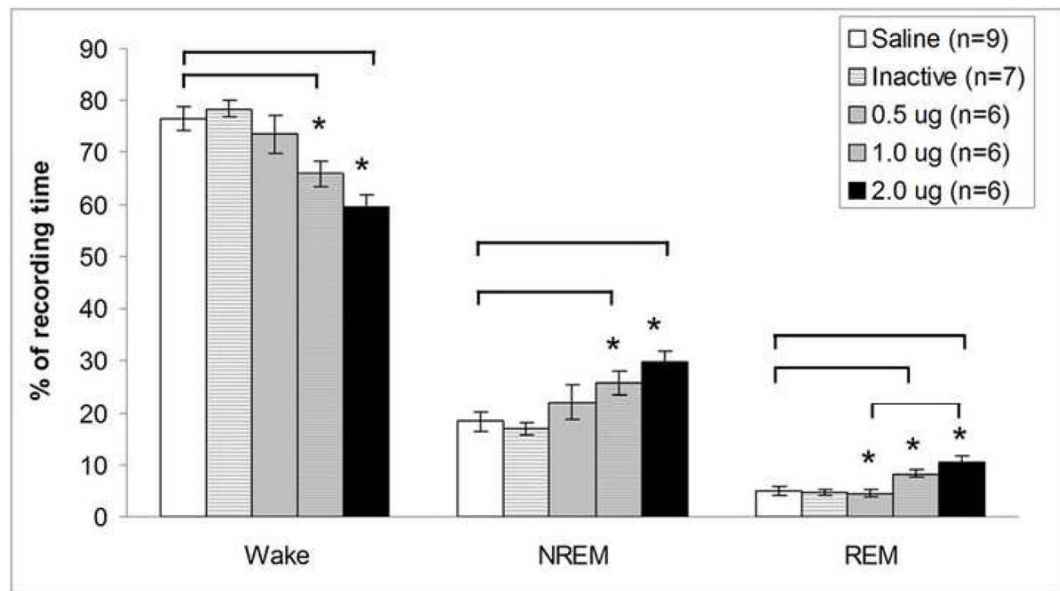


**Figure 1.** Total minutes of wake (a), NREM (b) and REM (c) (mean  $\pm$  SEM) per hour after intracerebroventricular delivery of either saline, inactive CART peptide or CART 55–102 (0.5  $\mu$ g, 1.0  $\mu$ g and 2.0  $\mu$ g) at the beginning of the customary sleep period. See text for details regarding statistical analysis. Asterisks indicate statistically significant differences across groups.



**Figure 2.**

Prototypical hypnograms of arousal states following icv delivery of either saline (top panel) or 2.0  $\mu\text{g}$  CART 55–102 (bottom). Administration was at the beginning of the hypnogram and the subsequent 6 hours of recording are depicted. Following saline (top panel), the animal is awake briefly and then transitions through NREM, REM and wake in a typical fashion. Following 2.0  $\mu\text{g}$  CART 55–102 (bottom panel), the animal remains awake for an extended period following by brief bouts of NREM and more wakefulness. Note the complete absence of REM sleep.



**Figure 3.**

The effect of CART 55–102 on sleep and wake states for the 12 hour customary wake (i.e. dark) commencing at CT 12 and twelve hours subsequent to its delivery at CT 0–1. Percent of the recording time spent in wake, NREM or REM sleep following delivery of saline, the inactive CART peptide, or varying concentrations of CART 55–102 is represented as mean  $\pm$  SEM. Asterisks indicate statistically significant differences across groups.