METABOLIC SIGNALS IN SLEEP REGULATION: THE ROLE OF CHOLECYSTOKININ

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A thesis for the degree of

DOCTOR OF PHYLOSOPHY

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To the memory of Ferenc Obál Jr.

Introduction

Most in the field of sleep research maintain that sleep is by the brain and for the brain. It is also evident that specific changes occur in the activities of many organs during sleep. Majority of these physiological adjustments are thought to be the direct consequence of sleep or the lack thereof. Other changes are not caused by sleep *per se* but are independent manifestations of the action of a common regulatory mechanism which they share with sleep/wakefulness. We posit that there is a third aspect of body-sleep interaction: physiological changes outside of the brain affect complex brain functions, including sleep. Part of these somatic changes is related to eating, adiposity or metabolism. Our long-term goal is to understand how metabolic status of the body affects brain in general and vigilance in particular. We aim to decipher the mechanisms involved in signaling to integrative sleep centers under various metabolic conditions. The present work focuses on one of the putative peripheral messengers involved in signaling between the body and sleep centers, the hormone cholecystokinin (CCK).

There is a strong bidirectional interaction between sleep/vigilance and metabolism/feeding. It has long been recognized that sleep is associated with characteristic changes in energy expenditure and metabolism. Acute metabolic changes in response to feeding or starvation as well as long-term metabolic shifts due to increased or decreased adiposity greatly affect the amount and the quality of sleep. We posit that hormones of the gastrointestinal (GI) system and adipose tissue play a key role in signaling for these adaptive sleep responses. Acute, transient changes in the amount and/or content of food profoundly affect sleep-wake activity in several species, including humans. In general, starvation induces a marked sleep loss while spontaneously or experimentally increased caloric intake leads to increased sleep. The regulation of sleep, feeding and metabolism overlaps on a structural level as several brain areas play a key role in the regulation of both sleep and metabolism/food intake. We propose that there is also overlap on a second, signaling level as well; as certain signaling mechanisms, particularly GI hormones, may be involved both in sleep and feeding/metabolism regulation. **Our broad hypothesis is that feeding-related GI hormones play a key role as metabolic signals in aligning vigilance with the current metabolic state of the body.**

Eating is followed by a characteristic postprandial behavioral sequence, called the satiety syndrome. Satiety syndrome entails the cessation of eating, transiently increased non-feeding activities such as grooming and exploration followed by reduced behavioral activity and social withdrawal ending with complete behavioral rest. Several GI hormones which are released after eating, e.g., CCK and gastric leptin, are known to suppress food intake and are thought to bring

about satiety. **The specific hypothesis tested in the present work is that CCK is a sleep-inducing hormone which contributes to signaling for postprandial sleep.**

There are two major, independent pools of CCK-producing cells, one in the GI tract and the other in the nervous system. Intestinal CCK serves as a GI hormone and paracrine agent while neuronal CCK is a neurotransmitter/neuromodulator. Various forms of CCK exist; in the brain of rats and mice, the predominant form is CCK octapeptide. Sulfated CCK octapeptide is the shortest form with full biological activity. Two G protein-coupled CCK receptor subtypes, CCK1 and CCK2 receptors, have been identified. Both receptors are expressed in the central and peripheral nervous system as well as in various organs of the GI system. Intestinal CCK is secreted postprandially in response to dietary fat and protein by enteroendocrine cells of the small intestines. CCK elicits a set of coordinated GI and behavioral responses characteristic of postprandial phase. CCK creates an alimentary environment favorable for fat and protein digestion by stimulating bile ejection and pancreatic enzyme secretion. CCK inhibits gastric emptying and secretion, thereby delaying the delivery of undigested chyme into the small intestines. These autonomic actions in the GI system during the post-meal period are complemented by postprandial behavioral responses, also triggered by CCK.

The best characterized behavioral effect of CCK is its suppressive action on feeding; CCK is thought to play a role in the short term regulation of feeding as a satiety hormone. CCK is released from the enteroendocrine cells after a meal and, by acting in a paracrine fashion, it binds to vagal CCK1 receptors to stimulate vagus afferents. This leads to the activation of nucleus tractus solitarius (NTS) – parabrachial nucleus (PBN) – ventromedial hypothalamus (VMH) circuit resulting in the inhibition of feeding. At the outset of our studies, several lines of evidence suggested that CCK might signal for postprandial sleep increases. CCK administration to fasted rats not only suppresses eating but it is followed by a complete sequence of behavioral events characteristic of rats after eating. This "satiety syndrome" terminates with resting. Since reduction of motor activity does not necessarily represent sleep, resting elicited by CCK might be a manifestation of behavioral sedation without sleep. Short episodes of sleep can often be observed after eating periods in rats. Supposing that postprandial sleep is a component of the behavioral manifestation of satiety, we postulated that resting observed after the injection of CCK may correspond to sleep. We set out to perform a series of experiments to determine the effects of CCK on sleep-wake activity and its role in postprandial sleep responses.

We tested the following specific hypotheses:

1. Systemic administration of CCK elicits sleep responses in rats.
2. Systemic but not central administration of CCK elicits sleep responses in rabbits.

3. The selective activation of CCK2 receptors by CCK tetrapeptide (CCK-4) or non-sulfated CCK octapeptide (CCK-8-NS) is not sufficient to induce sleep in rats.

4. The activation of CCK1 receptors is required for sleep responses in rats.

5. Sleep responses to systemically administered CCK are mediated by pancreatic insulin.

6. Intact CCK signaling on the CCK1 receptors is required for feeding-induced sleep responses.

Materials and Methods

Male New Zealand White rabbits and CFY, Wistar and Sprague-Dawley rats were used. Rabbits weighed 3-5 kg and the rats 260-420 g at the time of the experiments. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the respective institutional committees when applicable.

The animals were implanted with stainless steel screw electroencephalographic (EEG) electrodes and electromyographic electrodes for sleep recording and with a thermistor above the parietal cortex to record brain temperature. A guide cannula for intracerebroventricular (icv) injections was also implanted into the left lateral ventricle for rabbits. After surgeries, the animals were placed into individual sleep-recording cages inside sound-attenuated and temperature-controlled environmental chambers for a 2-week recovery and habituation period. During the habituation period and the sleep recordings, the implants were connected to amplifiers, the signals were filtered and digitized and collected by a computer. The animals were allowed to move freely in their home cages at all times, except during injections. In all experiments, a dark-light cycle of 12:12 h and ambient temperature of 21 - 24°C were maintained. Food and water were available *ad libitum*, unless noted otherwise.

Vigilance states were determined off-line by visually scoring the records in 10-30-s epochs or by an automatic analyzer. Wakefulness, non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) were distinguished using conventional criteria. In four experiments, spectral analysis of the EEG by fast-Fourier transformation (FFT) was also performed in delta frequency range. Delta wave activity of the EEG during NREMS (also called slow-wave activity, SWA) is a measure of sleep intensity.

Materials: Cholecystokinin octapeptide sulfate ester (synthesized by Botond Penke, University of Szeged for Experiment 1; purchased from Bachem Inc., Torrance, CA for Experiment 2 and Peninsula, Belmont, CA for Experiments 4 and 5), cholecystokinin tetrapeptide (Peninsula), nonsulfated cholecystokinin octapeptide (Peninsula), L-364,718 (Merck Research Laboratories,

Rahway, NJ), streptozotocin (Sigma, St. Louis, MO), insulin radioimmunoassay kit (Incstar Corp., Stillwater, MN). Injection volumes were 2 ml/kg for systemic treatments, and 25µl for intracerebroventricular (icv) injections in rabbits.

When feasible, repeated measures experimental design was applied. On the baseline day(s), normal sleep-wake activity, temperature and motor activity were recorded; the same animals were subjected to the experimental challenge on the test day.

Results

Experiment 1. *Effects of systemic injection of CCK in rats.*

Intraperitoneal (ip) injection of CCK elicited dose-dependent increases in NREMS, decreases in brain temperature (T_{br}) and suppressions in motor activity. Four µg/kg CCK was a subthreshold dose for all measured parameters. After the middle dose, 10 µg/kg CCK, there were significant increases in NREMS and decreases in T_{br} in the first h after the injection. NREMS increased at the expense of wakefulness, the amount of REMS was not affected. Increased NREMS was accompanied by suppressed motor activity. The highest dose of CCK, 50 µg/kg, caused a more than 200% increase in NREMS in the first hour. Motor activity was suppressed by ~73% and T_{br} dropped by ~0.9°C during this period. CCK also suppressed eating dose-dependently. Ten and 50 µg/kg reduced food intake by 45% and 63%, respectively; the lowest dose, 4 µg/kg CCK, did not have significant effects.

Experiment 2. *Effects of ip and icv injection of CCK in rabbits.*

The experiment was designed to test a) if the sleep-promoting effects of CCK are specific to rats or they are present in a second species and b) if central injection of CCK has also effects on sleep-wake activity.

Similar to the effects seen in rats, ip injection of CCK caused dose-dependent increases in NREMS and decreases in T_{br} in rabbits. Ten µg/kg CCK significantly decreased wakefulness and 40 µg/kg CCK significantly increased NREMS in the first h after injection. The lowest dose did not have significant effects on sleep or wakefulness. The somnogenic effects of CCK were accompanied by dose-dependent decreases in T_{br}.

In contrast to the effects of ip injections, icv administration of CCK did not cause any significant increase in NREMS in rabbits. Rather, 0.05 µg CCK reduced REMS across the 6-h recording period and 0.5 µg CCK reduced NREMS in the first h after the injection. There was a slight but significant decrease in T_{br} after the central injection of 0.05 and 2 µg CCK.

Experiment 3. *Effects of CCK2 receptor agonists in rats*.

The experiments aimed to determine if selective activation of CCK2 receptors is sufficient to elicit sleep responses characteristic of CCK. CCK2 receptors are present both in the central nervous system and in peripheral tissues. There are CCK2 receptor-selective CCK analogues available, such as CCK-8-NS and CCK-4. The affinities of CCK-8-NS and CCK-4 to CCK2 receptors are about 500-1,000 fold higher than to the CCK1 receptor.

In rats, ip injection of neither CCK-4 nor CCK-8-NS had significant effect on sleep or T_{br} in the first h; this is the time when the somnogenic and hypothermic effects of the sulfated CCK octapeptide are manifested.

Experiment 4. *Effects of CCK1 receptor antagonist on CCK-induced sleep in rats*.

The aim of the experiment was to determine if the activation of CCK1 receptors is necessary for the somnogenic effects of systemically administered CCK. CCK1 receptors are expressed in the brain, by neurons of the vagus nerve and by peripheral tissues. The food intake-suppressing effects of CCK are mediated by the activation of CCK1 receptors on vagus nerve terminals. L-364,718 is a widely-used and highly selective CCK1 receptor antagonist.

Intraperitoneal injection of L-364,718 alone did not have significant effects on spontaneous sleep, SWA and T_{br}. Ten µg/kg CCK, ip, elicited significant increases in NREMS and decreases in T_{br} in the first h after the injection. One hundred µg/kg L-364,718 significantly attenuated but did not completely block CCK-induced sleep; the same dose of L-364,718 prevented the hypothermic effects of CCK. Five hundred µg/kg of L-364,718 completely abolished CCK-induced sleep and hypothermic responses.

Experiment 5. *Effects of CCK in diabetic rats*.

CCK strongly stimulates pancreatic insulin secretion in rats. Insulin is known to enhance NREMS. In this set of experiments, we tested if the sleep-promoting effects of CCK are mediated by pancreatic insulin. We tested the effects of systemic injection of CCK on sleep in streptozotocin-induced diabetic rats.

Diabetic rats ate significantly more than controls on the baseline day. Intraperitoneal injection of 10 µg/kg CCK significantly suppressed feeding in both normal and diabetic rats by 53.6% and 37.5%, respectively. Baseline plasma insulin levels of diabetic rats were significantly lower than those of controls. In control animals, CCK significantly increased plasma insulin levels 5 and 15

min after injection. In diabetic rats, CCK did not have any significant effect on plasma insulin concentrations.

There were no significant differences in the baseline sleep-wake activity of control and diabetic rats. Intraperitoneal injections of CCK induced selective increases in NREMS in both the control and the diabetic groups in the first h after the injection. Ten µg/kg CCK doubled the amount of NREMS in the first h in normal rats; similar increases were observed in streptozotocin-pretreated animals. Fifty µg/kg CCK had a slightly more pronounced NREMS-promoting activity both in the control and diabetic rats.

Experiment 6. *Effects of CCK1 receptor antagonist on feeding-induced sleep*.

Increased feeding stimulates both CCK secretion and NREMS in rats. We tested the hypothesis that feeding-induced sleep responses are mediated by CCK acting on CCK1 receptors. To induce increased feeding, a fasting-refeeding paradigm was used.

Two baseline days were followed by 4 days of food deprivation and 2 days of refeeding. To induce fasting, food was removed at the end of the second baseline day (i.e., at dark onset of day 3); rat chow was returned to the animals 96 h later. The average weight loss during starvation was $13.2 \pm 1.0\%$ of the initial body weight. Two groups of rats were used. The control group received vehicle for L-364,718 on all 8 days. The experimental group was injected with vehicle on the baseline and food deprivation days and with 500 µg/kg L-364,718 on both refeeding days. Refeeding after the 4-d fasting period led to increased eating during the first night in both treatment groups. This increased eating was followed by elevated NREMS during the subsequent light period in the control group. Feeding-induced sleep responses were completely abolished by the CCK1 receptor antagonist treatment.

Discussion

Present results indicate that systemic injections of sulfated CCK octapeptide selectively and dose-dependently stimulate NREMS in rats and rabbits. Central injection of CCK in rabbits or systemic injection of CCK2 receptor agonists in rats did not have significant effects on sleep. The somnogenic effects of exogenously administered CCK as well as the sleep-inducing effects of refeeding after starvation were completely abolished by a selective CCK1 receptor antagonist. Systemic, but not central, administration of CCK elicited significant decreases in brain temperature, a response completely prevented by CCK1 receptor antagonist. The results are consistent with our hypothesis that CCK produced by the GI system in response to eating plays a

key role in eliciting postprandial sleep and thus in aligning vigilance to the acute feeding/metabolic status of the body.

Prior to our studies, only sparse and mainly indirect data were available concerning the effects of CCK on sleep. Subsequently, the sleep-promoting effects of CCK in rats were confirmed by independent laboratories. We also described the somnogenic actions of CCK in rabbits and later in mice thereby demonstrating that its sleep-promoting effects are not species specific. In all three species, the lowest somnogenic ip dose was 10 µg/kg. Although we did not observe any appreciable effects on duration of REMS in rats, rabbits or mice, there are reports that in parachlorophenylalanine-induced insomniac cats CCK restores REMS, and in normal rats CCK increases REMS frequency and decreases REMS latency.

We previously found that systemic injections of CCK elicit dose-dependent hypothermic responses in rats. These results were confirmed by the present experiments and replicated by independent laboratories. We extended these finding by showing that ip injection of CCK also produces dose-dependent hypothermic responses in rabbits. In rats, the dose-response relationships for the somnogenic, hypothermic and food intake-suppressing effects of CCK were similar. The effects of ip CCK on thermoregulation and sleep were also in the same dose range in rabbits. Our data indicate that the hypothermic response to CCK is mediated by the CCK1 receptor subtype. First, CCK2 receptor-selective CCK analogues, CCK-4 and CCK-8-NS, did not have hypothermic activities in our present and previous experiments in rats. Consistent with this observation, subcutaneous injection of the same analogues did not affect body temperature in mice. Second, the hypothermic effects of CCK were completely abolished by pretreatment with L-364,718, a selective CCK1 receptor antagonist.

The two main questions regarding the mechanism of CCK-induced sleep are related to the involvement of CCK1 vs. CCK2 receptor subtypes and the anatomical location of the target. Our results with CCK2 agonists and CCK1 receptor antagonist indicate that CCK2 receptor activation is not sufficient but CCK1 receptor activation is necessary for the somnogenic effects of CCK.

CCK-8-SE binds to both CCK receptor subtypes with equal affinity. If the somnogenic effects of CCK are due to the activation of CCK2 receptors then it is expected that equimolar amounts of sulfated CCK octapeptide, CCK-8-NS and CCK-4 would lead to similar sleep responses. This was not the case. The lowest effective somnogenic dose of systemically injected CCK-8-SE is 8.7 nmol/kg (10 µg/kg) in rats. In Experiment 3, the amount of NREMS did not increase in response to ip injection of 16.8-419.3 nmol/kg CCK-4 or 9.4-235.3 nmol/kg CCK-8-NS. These clearly show that the selective activation of CCK2 receptors is not sufficient to elicit somnogenic responses characteristic of CCK-8-SE.

L-364,718 is a selective antagonist of the CCK1 receptor. We found that 100 µg/kg L-364,718 nearly completely while 500 µg/kg completely abolished the sleep-inducing effects of CCK. This indicates that the activation of CCK1 receptors is necessary for the manifestation of sleep-inducing effects of ip administered CCK. The CCK1 antagonist did not affect spontaneous sleep in normally fed animals when given at dark onset suggesting that tonic activation of CCK1 receptors by endogenous CCK plays minimal role in maintaining spontaneous sleep at the beginning of the activity phase in rats.

Our findings that CCK2 receptor activation is not sufficient but CCK1 receptor activation is necessary for CCK-induced sleep responses do not rule out the possibility that the activation of CCK2 receptors also contributes to the sleep effects. The co-activation or sequential activation of CCK1 and CCK2 receptors may be necessary for the manifestation of the somnogenic effects of CCK. There are known effects of CCK that require the activation of both receptor subtypes, e.g., suppression of acetylcholine release from cerebral cortex or the potentiation of the anticonvulsive actions of morphine.

The present experiments with L-364,718 do not address the question of the site of the somnogenic effects of CCK. CCK1 receptors are present both in the brain and in the periphery. L-364,718 crosses the blood-brain barrier (BBB) after systemic injection and binds to both central as well as peripheral CCK1 receptors. Systemically injected CCK does not cross the BBB and likely acts on peripheral targets or brain structures that lack the BBB.

Regarding peripheral targets, we considered the possibility that the sleep effects of CCK are mediated through the release of another peripheral hormone stimulated by CCK. We considered insulin as a potential mediator of CCK's somnogenic action since CCK is a potent stimulator of insulin secretion and the effects of insulin on sleep and feeding are similar to those of CCK. To test the role of pancreatic insulin in the sleep-promoting action of CCK, we studied the effects of CCK in streptozotocin-diabetic rats. In line with the known stimulatory effects of CCK on insulin secretion, ip injection of CCK causes increases in plasma insulin levels in control rats but not in diabetics. In spite of the lack of insulin response, diabetic rats mounted similar sleep responses to CCK injection as normal animals indicating that insulin is not involved in the sleep actions of CCK.

We confirmed that increased feeding or postingestive satiety elicits postprandial sleep. The CCK1 receptor antagonist L-364,718 completely abolished the NREMS increases on refeeding days. This, together with the known increase of plasma CCK in response to feeding and our observation that L-364,718 abolishes exogenous CCK-induced sleep, strongly indicate a role of endogenously produced CCK in feeding-induced sleep responses.

The present work represents a segment of an ongoing broad project to test our model on the integration of metabolism and sleep (Fig.1). We hypothesize that in addition to the well-established wake-dependent homeostatic and suprachiasmatic nucleus (SCN)-driven circadian factors, metabolic signals also play a fundamental role in determining sleep-wake activity. We posit that CCK is such a metabolic signal. These signals may modulate the activity of arousal mechanisms or may modulate circadian influences by acting through the food-entrainable oscillator (FEO), an endogenous clock independent of SCN. Some signals trigger acute changes in sleep in response to short-term negative energy balance such as during starvation (ghrelin) or positive energy balance such as postprandial states (CCK, gastric leptin). Different signaling mechanisms set sleep amounts in response to long-term changes in adiposity (adipocyte-secreted leptin and TNF) or food availability (FEO). We hypothesize that both short- and long-term signals converge on a common integrative center in the hypothalamus. A ghrelin-neuropeptide Y-orexin circuit is thought to be a key component of this integrative center.

Fig. 1. Integration of metabolic and circadian signals in the regulation of sleep, wakefulness and feeding. SCN: suprachiasmatic nucleus, FEO: food-entrainable oscillator, TNF: tumor necrosis factor, PBN: parabrachial nucleus, NTS: nucleus tractus solitarius, NPY: neuropeptide Y
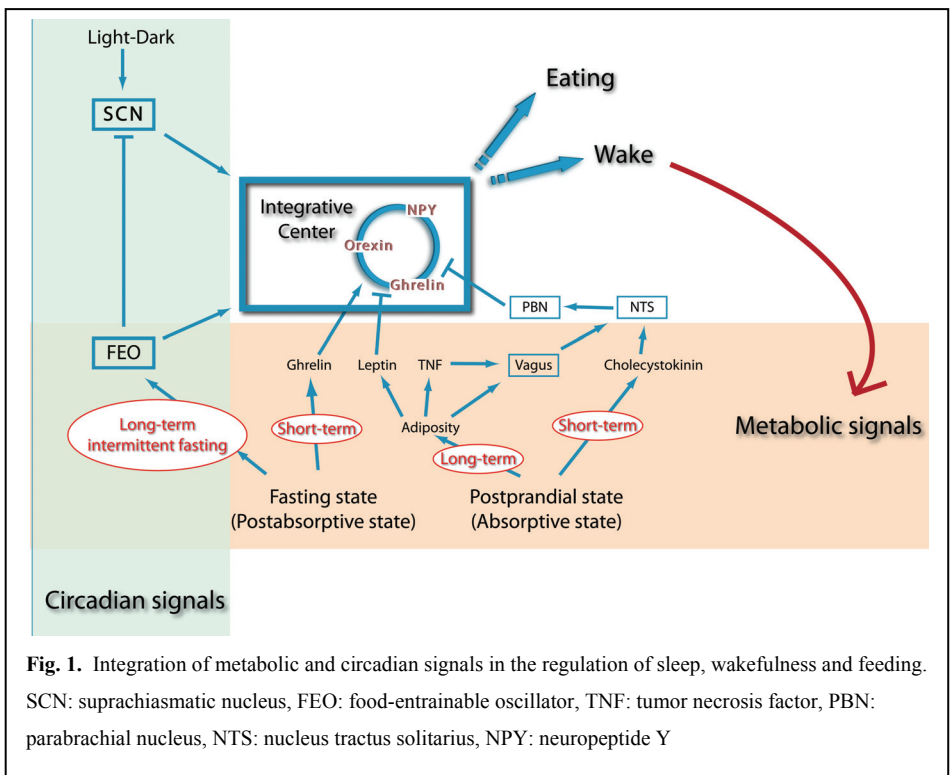
Circulating CCK may modulate the activity of this hypothalamic circuit by acting through a peripheral or central target or both. In Experiment 5, we ruled out pancreatic insulin as a possible peripheral mediator of CCK-induced sleep. Another potential peripheral target for CCK to induce sleep is the vagus nerve. Peripheral sensory nerve endings in the vagus abundantly express CCK1 and CCK2 receptors. Numerous effects of systemic CCK on brain functions are, indeed, mediated by vagal afferents. Further, sensory inputs from vagal afferents modulate sleep, the activation of vagus - NTS complex results in generation of NREMS. In our prior studies, however, surgical vagotomy did not prevent the somnogenic effects of ip injected CCK indicating that peripheral vagal CCK receptors are not a target for the somnogenic action of CCK.

The findings that lateral ventricular injection of CCK does not induce sleep in rabbits (Experiment 2) or in our previous studies in rats seemingly contradict a central target for CCK. The possibility, however, remains that there is a central target site which lacks the blood-brain barrier therefore can be reached by circulating CCK easily but less accessible for CCK injected into the lateral ventricle. We hypothesize that circulating CCK acts on CCK receptors within NTS to elicit sleep. CCK1 receptors are abundantly expressed in the NTS, mainly restricted to its medial subnucleus. The medial subnucleus is also rich in highly fenestrated capillaries similar to those in the area postrema. The lack of BBB makes neurons and CCK receptors in the NTS accessible to circulating CCK and other large blood-borne molecules. CCK by acting on NTS CCK1 receptors has known physiological actions such as eliciting satiety and modulating glutamate release from vagus afferents. The NTS may serve as a key interface between metabolic signals, both blood-born and vagus mediated, and sleep regulatory centers in the brain. NTS has extensive projections to the PBN. PBN itself is implicated in sleep regulation, most of its neurons show sleep-dependent activity pattern. From the PBN, dense projections arise to the VMH, DMH, posterior LH and preoptic hypothalamus, areas thought to play key role in the regulation of sleep and wakefulness.

In summary, we have shown that systemic injection of CCK elicits dose-dependent somnogenic and hypothermic responses in rats and rabbits. The sleep effects are accompanied by suppressed feeding and motor activity. Selective activation of CCK2 receptors is not sufficient to elicit the responses while the activation of CCK1 receptors is required suggesting CCK1 receptors as a primary target. Pancreatic insulin does not play a role in CCK-induced sleep and thermoregulatory responses. Eating-induced sleep is prevented by CCK1 receptor antagonist treatment indicating a role for CCK in the postprandial modulation of vigilance. Present results are consistent with the hypothesis that CCK is a component of a complex signaling mechanism which modulates sleep-wake activity according to the metabolic status of the body.

Acknowledgements

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