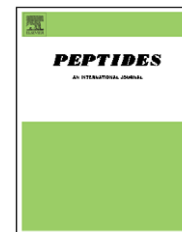


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## A tryptic hydrolysate from bovine milk $\alpha_{S1}$ -casein improves sleep in rats subjected to chronic mild stress

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### ABSTRACT

The putative effects of a tryptic bovine  $\alpha_{S1}$ -casein hydrolysate on stress-induced sleep disorders were investigated and their possible link with typical blood stress parameters such as plasma corticosterone concentrations and glycaemia was assessed. Rats were subjected to chronic stress in the form of environmental disturbances, while receiving an oral administration of the  $\alpha_{S1}$ -casein hydrolysate (CH). Chronic stress significantly reduced sleep duration in control rats during the first 2 days of the stress period, but stress-induced sleep disturbance was prevented in CH-treated rats. Indeed, CH administration allowed the maintenance of slow wave sleep (SWS) duration and even a slight increase in paradoxical sleep (PS) duration in treated rats. Results on plasma corticosterone concentrations and on glycemia values were inconclusive with respect to the implication of the HPA axis in this study. However, the protective effect of the  $\alpha_{S1}$ -casein hydrolysate on sleep during exposure to our chronic mild stress conditions may be mediated by modulation of the central adrenergic response.

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## 1. Introduction

Sleep disorders represent an important risk to health and recent studies have concluded that a sleep debt can facilitate the development of chronic metabolic disorders such as obesity, diabetes, and hypertension [36]. Environmental conditions can compromise sleep quality, but other factors including chronic stress [42,43], depression [2], shift work [18], abnormal dietary behavior (anorexia nervosa, obesity) [21], and malnutrition [10] are also associated with sleep disorders. Stressed individuals may find it difficult to fall asleep or remain asleep during the night. Conversely, insufficient sleep or frequent awakenings due to physiological problems or the demands of work may be stressful and affect mood and performance [1,4]. The pharmacological treatment of chronic

insomnia with standard hypnotics and sedative antidepressants is widely employed, but is not always fully efficient in restoring sleep. Poorer sleep quality with more awakenings, impaired or suppressed REM sleep is often reported during such treatment [31,45].

Cow's milk has long been considered a tranquilizing beverage with sleep-inducing properties. Sixty years ago, it was reported that adults consuming a meal of cornflakes and milk exhibited a stronger tendency toward uninterrupted sleep [20]. Using electroencephalography, other researchers found that sleep was significantly improved (longer and less broken) in older people fed a milk and cereal meal at bedtime, and that the action of milk was more effective with serial administration [6]. A positive impact on sleep has been associated with some milk protein fractions. This may

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originate from an energy-induced feeling of drowsiness [5,9], but also from an improvement in the ratio between tryptophan (Trp) and the pool of large neutral amino-acids (LNAA) in the blood, favoring the brain uptake of Trp, a precursor for serotonin synthesis that plays an important role in controlling sleep [7,30]. In addition, numerous bioactive peptides have been identified in milk proteins and may be released after enzymatic digestion in the gastrointestinal tract [23]. These include opioid and opioid antagonist peptides originating from milk proteins which very probably have a functional role by interacting with the endogenous opioid system [39]. They are good candidates for molecular players in the believed sedative effect of milk, but in many cases, there is still no evidence that they are released and activated under *in vivo* conditions. Findings that suggest a functional role for milk-derived opioid peptides as exogenous regulators in adult consumers have only been reported for the  $\beta$ -casomorphin group which indeed seems to participate in controlling various gastrointestinal functions [24,39].

More recently, while investigating whether popular views as to the sedative and calming properties of milk could be confirmed by the determination of bioactive peptides, it was discovered that a tryptic hydrolysate of bovine  $\alpha_{S1}$ -casein, and a decapeptide it contains ( $\alpha_{S1}$ -casein (f91–100) or  $\alpha$ -casozepine), displayed an anxiolytic-like profile in the conditioned defensive burying test and the elevated plus-maze following intravenous administration in rats [26]. Moreover, based on changes to blood pressure and cortisol levels measured in human subjects experiencing successive mental and physical stress situations, clinical study results clearly suggest the anti-stress properties of this hydrolysate following its oral administration [25]. Thus, in view of the acknowledged deleterious link between stress, anxiety and sleep, we hypothesized that this  $\alpha_{S1}$ -casein hydrolysate might also be implicated in the assumed hypnotic properties of milk, preventing or reducing the effects of stressful environmental conditions on sleep. In order to test this hypothesis, we investigated whether the oral intake of  $\alpha_{S1}$ -casein hydrolysate would reduce stress-induced sleep disorders. For this purpose, we subjected adult male rats to eight days of chronic mild stress [17] and measured the duration of total sleep (TS), slow wave sleep (SWS) and paradoxical sleep (PS) during this period. In addition, in order to investigate how far sleep changes were related to changes in the activity of the stress system, two physiological, stress-related blood parameters, plasma glucose and corticosterone concentrations, were monitored.

## 2. Materials and methods

### 2.1. Test product and animals

Bovine  $\alpha_{S1}$ -casein tryptic hydrolysate (lactium<sup>TM</sup>) which will be referred to as CH in this article, was supplied by Ingredia (Arras, France). The control substance consisted of total bovine milk proteins. These two products were mixed with an appetitive liquid vehicle product (Fortimel, Nutricia, France) so that they could be administered orally in the rats. During all our experiments, both CH and control rats were

treated daily at 18:00 h by oral administration (15 mg/kg). The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC), on the care and use of laboratory animals.

Male Wistar rats (Harlan, France), weighing 240–260 g at their arrival in our facility, were kept under standard conditions of temperature ( $22 \pm 1^\circ\text{C}$ ) and under a reversed 12 h light–dark cycle (lights on at 18:00 h). They were housed in individual cylindrical Plexiglas cages with an opening at the top to enable electroencephalographic recordings [28] and blood sampling. Standard food pellets (AO4, SAFE) and water were available *ad libitum* throughout the experimental period. The rats were handled twice a day by the same experimenter and particular care was taken to limit any kinds of external stress (variations in light, noise or odor). The animals were allowed to adapt to these conditions and to the sleep recording procedure for at least three weeks before the beginning of the experiment. They were also accustomed to the oral administration of 0.3 ml of a vehicle, each day at 18:00 h.

### 2.2. Implantation of EEG electrodes

After adaptation to the laboratory conditions the rats were implanted with cortical electrodes for EEG recordings. They were anaesthetized with a mixture of ketamine (75 mg/kg, Imalgène 1000) and xylazine (1 mg/kg, 1% Rompun) and placed on a stereotactic frame. Two silver wire (Phymep) EEG electrodes soldered onto a female connector (male SMC base for coaxial connectors) were fixed in place. The cortical electrode, which terminated with a 0.5 mm diameter sphere, was inserted via a hole in the skull until it came into contact with the dura mater. This electrode was positioned 3 mm lateral to the sagittal suture and 4 mm anterior to the bregma. The second electrode, used as an earth electrode, was positioned subcutaneously. Four jewellery screws were then fixed lateral to the skull and served to anchor the electrodes and connector with dental cement (Dentalon Plus, Kulzer-Heraeus). No muscle electrodes were used because, as shown by Danguir, the derivations employed ensured a clear distinction between the three main vigilance stages: wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) [9,27].

### 2.3. Implantation of a jugular catheter

Animals were anaesthetized with a mixture of ketamine (75 mg/kg, Imalgène 1000) and xylazine (1 mg/kg, 1% Rompun) and a 70 mm long catheter (602–135 Silastic Brand from Dow Corning, Medical Products, Midland, MI, USA) was inserted into the right external jugular vein some 5 mm before it dives under the clavicle [28] and then pushed down the vena cava. The catheter was not introduced using a hard guide, but filled with physiologic saline and pushed gently back and forth until it found its way into the vena cava. The catheter was then secured to the vein and passed subcutaneously along the shoulder and the neck to reach the skull, where it was attached with screws and dental acrylic (Dentalon plus from Heraeus Kulzer, Dormagen, Germany) and then filled with viscous 30% polyvinyl pyrrolidone (Prolabo) to prevent occlusion by blood.

## 2.4. Experimental designs

### 2.4.1. First experiment

The rats were implanted with electrodes for EEG recordings and accustomed to the recording procedure. The surgical procedure induced a transient decline in body weight, but preoperative weight was recovered within 3 days. After at least 1 week of recovery, basal sleep was recorded over two consecutive days. The rats were then separated into three groups ( $n = 8$  in each group). In the first one, the rats were treated with the  $\alpha_{S1}$ -casein tryptic hydrolysate (CH stressed group) while in the second one, the rats received the control substance (control stressed group). In these two groups, the treatment was given per os for 8 days, at the same time as the rats were subjected to chronic mild stress. The third group was used to test the impact of CH treatment on sleep under normal conditions (CH-unstressed group).

EEG recordings were performed during 16 h, from 6 pm (lights on) to 10 a.m., at days 1, 2, 4 and 8 of the treatment period. The recording period thus included the entire light period, during which the rats use to develop most of their sleeping time. Furthermore, our main goal was to focus on the sleep response to ingestion of CH and we could anticipate that most of the effect would occur between 1 and 12 h after ingestion.

### 2.4.2. Second experiment

The same experimental design was applied to a second group of rats ( $n = 6$  in each group) chronically implanted with a jugular catheter for remote blood sampling. They were allowed to recover for at least a week before the start of the mild stress period. The animals were habituated to handling during daily inspections of their catheters. Blood (100  $\mu$ l) was collected at 11:00, 15:00, 17:30, 19:00 and 21:30 h on three consecutive days: the day before and then the first 2 days of the mild stress period, via the jugular catheter. During blood collection, the catheters were continuously connected to a syringe filled with heparinized saline solution (100 IU/ml) with polyethylene tubing. Rats were injected with 100  $\mu$ l of a solution of 9% NaCl and 6% citrate after each collection. Glucose levels were determined immediately on whole blood using a Glucotrend 2 (AccuChek system, Roche, limit of sensitivity: 0.6 mmol/l). Blood samples were mixed with an EDTA-Trasyol (Bayer AG, Leverkusen, Germany) solution (25%), centrifuged at  $3000 \times g$  (4 °C) for 15 min and frozen until analysis. Plasma corticosterone levels were determined using a commercially available radioimmunoassay kit (MP Biomedicals, Orangeburg, NY).

### 2.4.3. Mild stress procedure

Rats were subjected to disturbances of environmental parameters during an 8-day procedure when we randomly combined various environmental stressors such as sonorous disturbances (approximately 85 dB, produced by an untuned radio), light-dark cycle disturbances (ten 5 min long episodes during a 24-h period) and a different tilt to the cage every 12 h (approximately 30° from upright).

## 2.5. Sleep recordings and scoring

A flexible electrical wire was screwed to the skull connector by means of a female SMC-plus for coaxial connectors and

plugged into an electrical swivel connector (Air precision, Le Plessis-Robinson, France) in order to transmit the EEG signal to an amplifying system (10 000 $\times$ ) (Exp-EEG1, extracellular amplifier for EEG measurement, Experimetria Ltd., Budapest, Hungary). The amplified signal was then transmitted to a data acquisition board (DAS 1800, Keithley Instruments, Palaiseau, France) plugged into a personal computer. Data was acquired from eight rats simultaneously over a period of 16 h, from 18:00 to 10:00 h the next day, under the control of a PC program written in the laboratory using ASYST V4.1 (Keithley Instruments). The amplified signals were digitized at a frequency of 100 Hz, filtered below 1 Hz and above 40 Hz and saved on the hard disk at 10 s intervals. All measurements of wakefulness (W), slow wave sleep (SWS) and paradoxical sleep (PS) were made by visual inspection of the graphic replay of data files on a computer screen by two "blind" independent observers, with the help of a semi-automatic analysis program. Briefly, the observers visually discriminated between 20 and 30 characteristic 10 s periods for W, SWS, and PS, from which the computer program defined the specific power spectra of W, SWS and PS episodes. Sleep stages were then computed automatically by the program by comparing the power spectrum for each 10 s period with the recorded specific power spectra (identity  $\sim 5\%$ ). The observers completed their sleep pattern analysis by defining visually the 10 s sleep episodes for which the power spectrum differed by more than 5% from the typical power spectrum ( $\sim 5$ –8% of episodes). The assignment of sleep stages followed standard criteria. With respect to EEG patterns, SWS episodes of less than 20 s within a W episode were not distinguished from wakefulness and vice versa, W episodes of less than 20 s within a period of SWS were not distinguished from SWS (performed automatically by the analytical program). For each 16-h period, analysis of the sleep-wake cycle provided EEG recordings of the time spent in wakefulness, SWS and PS.

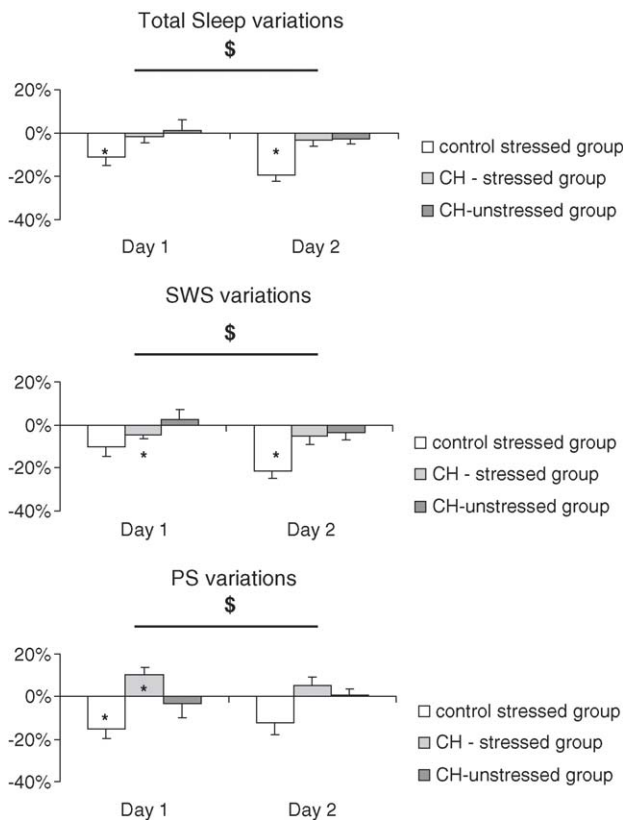
## 2.6. Statistical data analysis

Data are reported as mean  $\pm$  standard error of the mean (S.E.M.). Paired data analysis (Student's *t*-test for paired data) was performed on EEG, blood glucose and plasma corticosterone values. Moreover, when time effect was considered, differences between groups were determined by a mixed procedure (Proc mixed, SAS version 6.11, model chosen according to BIC parameters) using group, time and group  $\times$  time effects. The level of significance was set at  $P < 0.05$ .

## 3. Results

### 3.1. Short-term effects of mild stress and CH on sleep parameters

Variations in sleep durations (total sleep, SWS and PS) between baseline and the first 2 days of the mild stress period during the first experiment are shown in Fig. 1. The mild stress period induced a dramatic fall in total sleep duration in control stressed animals. This reduction was significant as early as the first day ( $-11\%$ ) and further increased to  $-20\%$  on the second day. More precisely, this reduction resulted from a decrease in

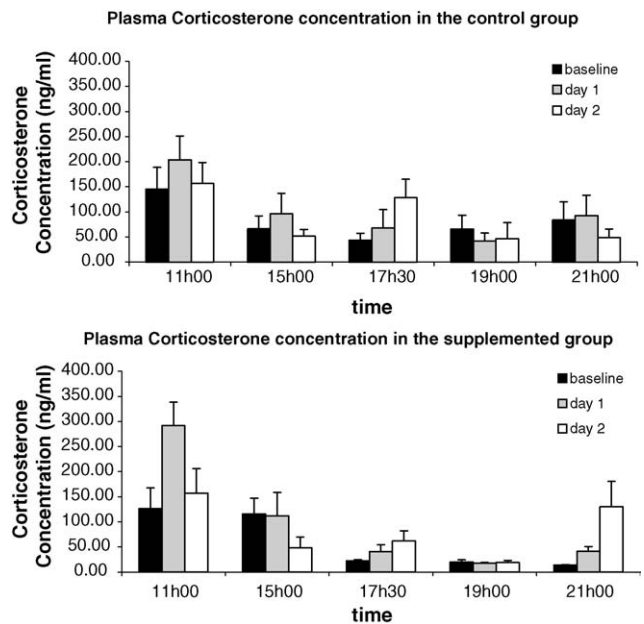


**Fig. 1 – Differences in sleep patterns between baseline and the first two days of treatment in the control stressed, CH-stressed and CH-unstressed groups. \*Data significantly different from 0; \$Significant between-group differences at  $P < 0.05$ .**

both SWS (-10% on day 1, -22% on day 2) and PS (-15% on day 1, -12% on day 2, NS). In the CH-stressed group, the deleterious action of stress on sleep was not observed to such an extent. Sleep decreased by only 2% during day 1 (NS) and by 4% during day 2 (NS). The results obtained regarding sleep components in the CH-stressed group further demonstrated that the slight decrease in SWS was only significant during day 1 and that there was even a surprising increase in PS over these first 2 days (significant at day 1). Between-group comparisons showed that the reduction in total sleep duration observed in the control stressed group was greater than that observed in the CH-stressed group (group  $P = 0.002$ , time  $P = 0.0003$ , group  $\times$  time  $P = 0.0087$ ), with significantly lower SWS (group  $P = 0.0141$ , time  $P = 0.0005$ , group  $\times$  time  $P = 0.0295$ ) and PS (group  $P = 0.001$ , time  $P = 0.6515$ , group  $\times$  time  $P = 0.0047$ ) durations. Moreover, Fig. 1 shows that CH treatment did not affect sleep in unstressed rats, indicating that the resistance to stress of the CH-treated rats is not due to a sedative effect of the CH treatment per se.

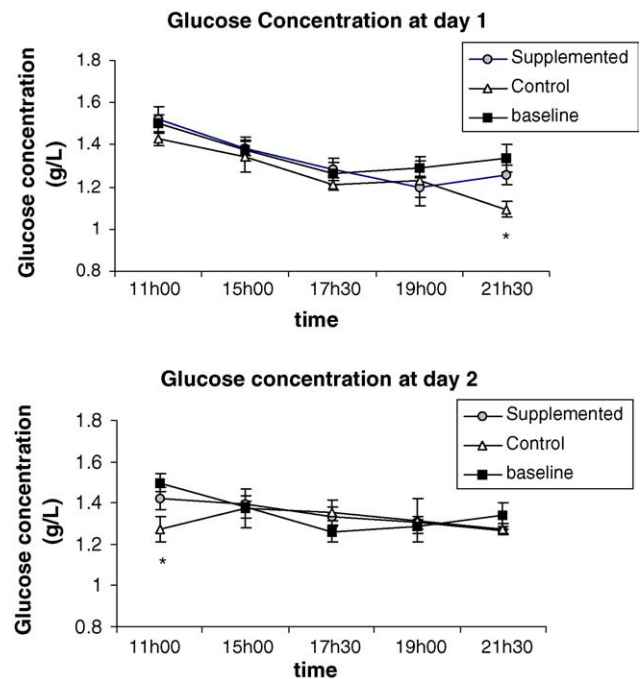
**3.2. Short-term effects of mild stress and CH on blood parameters**

Plasma corticosterone and blood glucose levels determined in the second experiment at baseline and during the first 2 days of the mild stress period are presented in Figs. 2 and 3.



**Fig. 2 – Plasma corticosterone levels (ng/ml) at baseline and during the first 2 days of mild stress: (a) control group and (b) CH group.**

No significant differences in corticosterone concentrations between baseline and the first 2 days of mild stress were observed in both the control and CH groups. Indeed, no significant time effect could be identified besides the usual



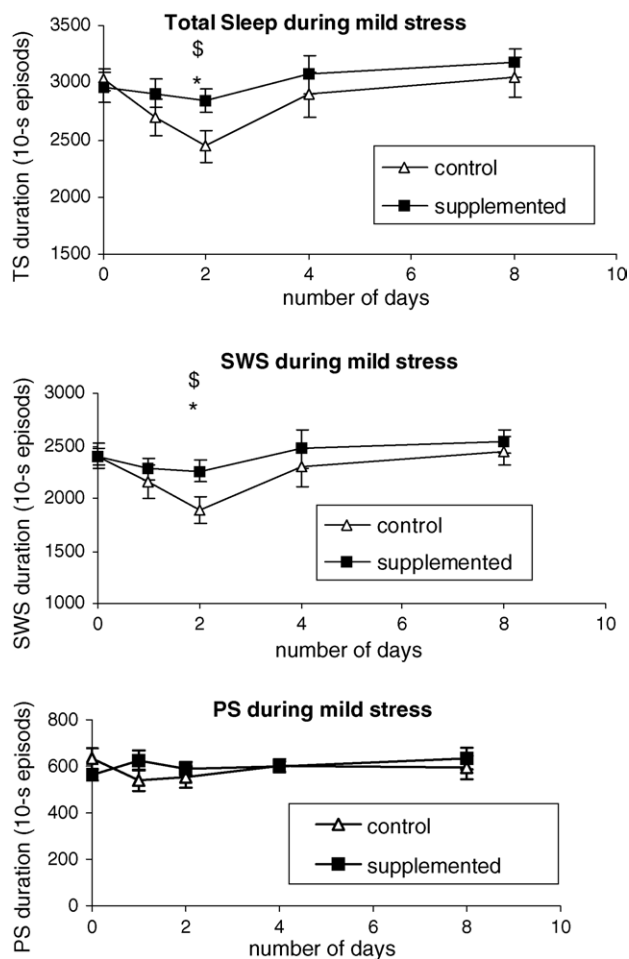
**Fig. 3 – Plasma glucose levels (g/l) at baseline and during the first 2 days of mild stress: (a) first day and (b) second day. \*Significant intragroup differences from baseline ( $P < 0.05$ ).**

circadian variations of plasma corticosterone. Between-group differences were not significant neither.

Blood glucose levels in the control group were significantly lower than baseline after 18:00:h during the first day of mild stress, whereas no significant changes were observed in the CH group. At the beginning of the second day, these levels were still reduced in the control group but thereafter progressively caught up to no longer differ from baseline. No between-group differences were found.

### 3.3. Long-term effects on sleep parameters

The amounts of total sleep, slow wave sleep and paradoxical sleep during the entire mild stress period are shown in Fig. 4. First of all, these data confirm a significant decrease in total sleep duration in the control group, mostly due to a significant shortening in the duration of SWS during day 2 of the mild stress procedure. They also confirm the preservation of sleep in CH treated rats. Moreover, despite pursuit of the mild stress procedure, sleep gradually recovered in the control group and was no longer significantly affected after 4 days.



**Fig. 4 – Evolution of sleep patterns during the mild stress procedure in control and CH-treated groups. Significant intragroup differences from baseline; \$Significant between-group differences at  $P < 0.05$ .**

## 4. Discussion

The aim of our study was to establish a model of stress-induced sleep disturbances in order to test the ability of the bovine  $\alpha_{S1}$ -casein tryptic hydrolysate (CH) to antagonize sleep disorders. In humans, stress and sleep disturbances are often related [12,19]. In rats, it has already been shown that chronic stress induces a marked sleep reduction [17]. However, this phenomenon is transient. Effectively, it was shown that sleep during the light hours was decreased throughout the stress period, while sleep during the dark hours increased within the first day of stress consequently daily sleep had returned to pre-stress levels by the third day of stress. In accordance with these results, our procedure disturbed sleep composition in control stressed rats as early as the first day of the chronic mild stress procedure, with reductions in both SWS and PS. Sleep disturbances were further enhanced during the second day, but began to decrease on day three and after day 4, no difference with the pre-stress period persist. However, it was not possible to state whether these findings reflected adaptation by rats to mild stress over time or whether they were indicative of sleep organization adaptation throughout the day, in order to respect sleep homeostasis despite stressful events. As expected, we therefore obtained an interesting model of stress-induced sleep impairment during the first 2 days of our chronic mild stress procedure.

The main finding of this study was the protective effect of oral CH administration with respect to sleep duration. Indeed, total sleep duration in CH-treated rats was not reduced by the stress procedure and was significantly higher than in control animals. In addition, we observed that CH did not affect sleep in unstressed rats, but prevented sleep disturbance in stressed rats. Consequently, it can be said that this effect is a component of the anxiolytic properties of CH, with sleep-disorders being considered as anxiety-related disorders, rather than a sedative effect by itself. This protective effect resulted from two phenomena: first, the maintenance of slow wave sleep, and second, a slight increase in paradoxical sleep. As expected, the total sleep, SWS and PS time course (data not shown) clearly indicated that the effects of CH developed between 1 and ~10 h after ingestion. These data should first of all be acknowledged as a single, rare result showing the specific behavioral impact of an ingested hydrolysate. Above all, the sleep promoting or sleep protecting effect of the bovine  $\alpha_{S1}$ -casein tryptic hydrolysate tested under chronic stress conditions provides a possible explanation for the assumed properties of milk, as well as offering a potential use of this hydrolysate in the management of human stress and sleep disorders, which is not surprising in view of the suggested anxiolytic-like activity of this substance.

During the second part of the study, we investigated the effect of CH administration on the stress system by monitoring plasma levels of corticosterone, a marker of hypothalamic-pituitary-adrenal axis mactivity, and blood glucose level, which is currently considered as a stress-sensitive parameter, during the early days of the stress procedure. We did not observe any significant change to plasma corticosterone levels in response to the mild stress procedure. Indeed, previous studies on sleep-stress interactions had highlighted the key role of both components of the stress system: the hypotha-

lamic-pituitary-adrenal axis and the sympathetic system. While both are down-regulated during sleep [34,44], it has been shown that their activity relates positively to the degree of objective sleep disturbance [43]. On the other hand, experimental sleep disruption (frequent awakenings) is associated with a significant rise in plasma cortisol levels in humans [35]. However, caution should be exercised when interpreting these results. Although chronic stress and poor sleep have been associated with high corticosterone levels in some studies [16,41], no impact is observed in others [37]. Results seem to depend on the rat strain [3] and have been inconclusive with the Wistar strain. Moreover, the mild nature of stressors may also contribute to the weakness of the HPA axis response. Some investigators have demonstrated a correlation between stressor intensity and corticosterone levels [14,29]. It is therefore possible that the low susceptibility to stress of Wistar rats and the marked inter- and intra-individual response variability interacted with the low intensity of the stressors associated in our paradigm to prevent the occurrence of any significant variations in corticosterone levels. Nonetheless, we observed an interesting and significant reduction in plasma glucose levels in control-stressed animals but not in CH-treated-stressed animals during the first day of the chronic stress procedure, suggesting that glycaemia may be a more sensitive marker of the stress system activation than plasma corticosterone. As a result, we hypothesize that our mild stress conditions probably induced stress system responses and sleep impairments through a slight but repeated central and sympathetic adrenergic activation rather than through activation of the HPA axis. Therefore, the model we used should be considered as a model of stress-induced anxiety, and anxiety-induced insomnia. In this model, CH effects could be mediated by modulation of intensity and duration of the adrenergic activation, for instance through the opiate or gabaergic systems.

The precise mechanisms underlying the effects of  $\alpha_{S1}$ -casein tryptic hydrolysate on stress-induced sleep disturbances still need to be determined. Target sites, precise active fragment(s) of the hydrolysate and pathways mediating this action must be elucidated. When the hydrolysate is orally administered, some fragments that may be resistant to digestion and absorption processes could be candidates as bioactive components. Some bioactive peptides (such as atypical opioid peptides) have been shown to be encrypted in bovine  $\alpha_{S1}$ -casein and are considered as good candidates. They have been shown to be  $\delta$ -selective receptors ligands [22,24] and could as such be candidates for a central nervous function like stress adaptation, but evidences still lack regarding the release or functional role of these peptides in vivo [38]. On the other hand, as mentioned in the general introduction, a peptide called  $\alpha$ -casozepine has been identified in the hydrolysate and shown to exhibit affinity for the GABA<sub>A</sub> receptor as well as anxiolytic activity following intravenous administration [26]. The effects of  $\alpha_{S1}$ -casein tryptic hydrolysate CH thus may be due to this active decapeptide, which needs to be explored in a further study so as to clarify the possible functional role of this peptide in vivo following oral administration.

Bioactive and intact peptides may enter peripheral blood and exert systemic effects, or may exert local effects in the

gastrointestinal tract. To be addressed, this question requires to assay bioactive candidates or various other potential fragments in the blood, which remains a complex and unsolved problem at present. Until now, evidences suggest that peptides larger than tripeptides cannot pass through the adult digestive endothelium in significant amounts. Duodenal digestion involves the cleavage of numerous peptide linkages by pancreatic endopeptidases and exopeptidases. The neonatal intestine clearly appears to be more permeable to bioactive peptides, as has been shown with  $\beta$ -casomorphins [32,40], but in adults the intestinal mucosa acts as an efficient barrier between luminal contents and the systemic circulation [15]. However, bioactive peptides were found in the blood of adults rats or humans after ingestion [8,11], and some studies have reported enhanced permeability of the intestinal barrier under various stress conditions [13,33]. These results, originally directed to explain the pathogen and toxin sensitivity of stressed subjects, may provide an explanation for the stress-induced effect of the hydrolysate: active molecules may not be able to pass through the intestinal barrier and reach their target in sufficient amounts unless stressful conditions are met. As a consequence, they could survive enzymatic attack in the plasma and act, even in small amounts, at any potential site of action in the organism. In addition to a potential systemic or even central direct action, the effect on adult rats could be further mediated via subepithelial receptors or specific luminal binding sites in the brush border membrane. In that prospect, it could be very interesting to investigate the role of the vagus nerve, which is a possible relay for signal transfer at a central level.

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## 5. Conclusions

To date, few studies have demonstrated an effect of orally administered food-derived peptides on an adult organism, and as far as we know, none has recognized such a global behavioral effect of an orally administered peptide. This study highlights for the first time the sleep promoting effect of bovine  $\alpha_{S1}$ -casein tryptic hydrolysate treatment during chronic stress, a result which may open new perspectives on the mechanisms by which food-derived bioactive peptides act under stress.

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