Looking at Alpha Casozepine
Clinician's Notes - Dr Erik Johnson

http://johnsonvet.com/dementia/#p=10

http://johnsonvet.com/dementia/#p=12

Alpha-Casozepine (Zylkene)

15mg/kg Alpha-casozepine

http://johnsonvet.com/dementia/#p=14

Researcher: Miclo, Perrin benzodiazepine-like activity
Researcher: Mizushige T, Sawashi Y, Yamada A anxiolytic-like

https://pubs.acs.org/doi/full/10.1021/jf104089c
α-Casozepine is a peptide, corresponding to the sequence 91−100 of the bovine αs1-casein, displaying anxiolytic activity in the rat. The αs1-casein tryptic hydrolysate containing this peptide decreases stress effects after oral administration in various species including man. Therefore, the stability of this peptide toward gastric and pancreatic proteases has been assessed by using pepsin, chymotrypsin/trypsin, Corolase PP, pepsin followed by chymotrypsin/trypsin or pepsin followed by Corolase PP. α-Casozepine was slowly degraded by chymotrypsin, much more sensitive to pepsin and Corolase PP but not completely destroyed after 4 h kinetics. The bonds in the region 91 to 95 of the α-casozepine were totally resistant to hydrolysis by all studied proteases. Surprisingly, a fragment, corresponding to the sequence 91−97 and found in all the hydrolysis media in significant amount, possessed an anxiolytic activity in three behavioral tests measuring this parameter. This peptide could participate in the in vivo activity of α-casozepine.

UNDERSTANDING C3–COLOSTRUM CALMING COMPLEX

INTRODUCTION

The Composure product distributed by VetriScience Laboratories has been shown to be an effective formulation which provides a calming and anxiolytic response in stressed dogs and cats. The positive effect of the product has been demonstrated in several controlled field studies and from observations made by of both veterinarians and pet owners. Composure contains Thiamine (Vitamin B1) , L-Theanine and a colostrum derived ingredient called Colostrum Calming Complex which is abbreviated as C3. These active agents work synergistically to counter the effects of environmental stressors.

This piece will focus on the C3 ingredient; how it is produced, its composition and its complex actions to produce a calming effect in dogs and cats.

How is C3 Produced

C3 is derived from bovine colostrum which is collected from the first 12 hours of milk production after birth of the calf. The cows from which the colostrum is collected are fed a carefully designed scientific diet which not only ensures the health of the mother but also that the colostrum produced is of high quality, consistent in composition and nutritionally complete. This diet is free of synthetic hormones, pesticides and antibiotics. Additionally, all herds used to source the C3 ingredient are maintained in the U.S.

C3 is produced under carefully supervised cGMP (current good manufacturing practices) The C3 is truly a unique product that is produced by a microfiltration process that isolates a consistent blend of highly bioactive ingredients from which most of the lactose and fat have been removed. It is done under low heat and sterile conditions so as to preserve the structure and biologic activity of the components. The consistency and activity of the ingredients are confirmed by HPLC and cell culture techniques.

What is the Composition of C3

C3 contains a wide range of bioactive molecules which empower the body's natural abilities to handle environmental stressors.

C3 's key components include:

- Bioactive peptides and proteins
- Immunoglobulins
- Calcium calmodulin
- Glycoproteins
- Monosaccharides
- Growth and Transfer Factors
- Lactoalbumins
- Lactoferrins
- Oligiosaccharides
- Proline rich polypepides (PRP)
- Sialic Acid

How does C3 Work

The colostrum calming complex C3 supports brain activity, relaxation and cognitive function. The biologically active components present in C3 are diverse and complex. At this point, most of the mechanisms of action of C3 are unproven. The effects of the Calming Colostrum Complex however, have been tested and authenticated.

One of the active agents in C3 is a decapeptide that has a similar mode of action as alpha casozepine. This is proposed to work by binding to the benzodiazepine receptors to enhance the effect of GABA. GABA is an inhibitory neurotransmitter and by increasing GABA's activity, the brain is less reactive. There are several types of GABA receptors. The first is the most common and mediates sedation and tolerance. C3 has been shown to be non-sedative and tolerance doesn't occur with long term usage. A second receptor is present in the hippocampus as well as in the spinal cord. Activation of these receptors is important for cognition and anxiolytic actions. This is where C3 has its most important effect. The activation of these receptors creates a non-sedative but relaxed state of mind but at the same time doesn't induce muscle relaxation or memory loss. This creates a calm state while the dog or cat remains alert and responsive. C3 also contains between 50-100 oligosaccharides that synergistically enhance the effect of the decapeptide.

Another active component of C3 is Sialic Acid (N-acetylneuraminic Acid) . Sialic Acid has been shown to modulate the immune system and support cellular communication. It is linked to improved memory and cognition. Sialic acid is easily absorbed and abundant in colostrum protein.

Proline rich polypeptides are associated with enhancing mood and cognitive abilities. These polypeptides are best sourced through colostrum.

CONCLUSION

C3 is a safe and effective calming agent countering the effects of environmental stressors in companion animals. C3 has been shown to be part of an anxiety reduction protocol which produces enhanced cognition without sedation. Its use produces a repeatable and reliable effect

and can be used as part of a comprehensive behavior modification treatment plan. When combined with the other ingredients in Composure, C3 can positively impact the quality of life for animals and their owners.

References for C3

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Modulation of Cerebral Activity Induced by α-casozepine, a Benzodiazepine-like Peptide Derived from Bovine Casein

1. **Simon Benoit**1,2,
2. **Catherine Chaumontet**1,
3. **Céline Cakir-Kiefer**2,
4. **Daniel Tomé**1**and**
5. **Laurent Miclo**2

+Author Affiliations

1. [1]UMR Nutrition Physiology and Ingestive Behavior (PNCA), AgroParisTech, INRA, University Paris-Saclay, Paris, France
2. [2]UR AFPA-INRA, Université de Lorraine-Nancy, Vandoeuvre-lès-Nancy, France

Mol Pain. 2005 Aug 15;1:22.

Genetic alteration of anxiety and stress-like behavior in mice lacking CaMKIV.

Shum FW[1], Ko SW, Lee YS, Kaang BK, Zhuo M.

Author information

Abstract

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A proline-rich polypeptide complex and its nonapeptide fragment inhibit nitric oxide production induced in mice

AgnieszkaZabłockaMariaJanuszJózefaMacałaJózefLisowski https://doi.org/10.1016/j.regpep.2004.07.024Get rights and content

Dietary sialic acid supplementation improves learning and memory in piglets1,2,3

1. **Bing Wang,**
2. **Bing Yu,**
3. **Muhsin Karim,**

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+Author Affiliations

1. [1]From the Human Nutrition Unit, School of Molecular and Microbial Biosciences (BW, MK, HH, YS, and JB-M), Department of Molecular and Clinical Genetics, Royal Prince Alfred Hospital (BY), Faculty of Veterinary Science (PMcG), University of Sydney, Sydney, NWS, Australia; Department of Statistics, Macquarie University, Sydney, NSW, Australia (PP); and Centre for Behavioural Biology, Department of Clinical Veterinary Science University of Bristol, Bristol, United Kingdom (SH)

Roger Kendall PhD

Dr. Kendall received his undergraduate degree in Chemistry from the University of Vermont in 1965 and went on to obtain his PhD in Organic/Biochemistry in 1970 from Penn State University. He has held teaching positions at University of Bridgeport and Ambassador University and presently holds seven U.S. Patents and two European Patents, six of them on Dimethylglycine. He has worked for over 35 years in the field of nutritional research and product development, and is considered to be the leading expert in the biochemistry and therapeutic applications of DMG.

Elizabeth DeLomba, DVM MBA

For over 26 years, Dr. DeLomba has combined private clinical companion animal practice with corporate veterinary consulting. She has over 13 years of private clinical companion animal practice experience combined with more than 12 years of corporate veterinary pharmacy experience. Dr. DeLomba holds a BA in Biological Sciences from Smith College, a DVM in Veterinary Medicine from Michigan State University, and a MBA from Villanova University.

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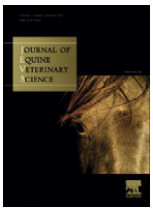

Original Research

Calming Benefit of Short-term Alpha-Casozepine Supplementation during Acclimation to Domestic Environment and Basic Ground Training of Adult Semi-Feral Ponies

Sue M. McDonnell PhD [a], Jaime Miller AS CAHT [a], Wendy Vaala VMD, dip ACVIM [b]

[a] *Havemeyer Equine Behavior Lab, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA*
[b] *Merck Animal Health, One Merck Drive, Whitehouse Station, NJ*

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ABSTRACT

To evaluate potential calming effects of alpha-casozepine on horses, we blindly compared behavior and training efficiency of adult semi-feral ponies treated with either alpha-casozepine or control supplement during transition to domestic management and handling. Six ponies (three matched pairs) aged 2 to 8 years that had been reared and kept since birth under semi-feral social and environmental conditions were given either alpha-casozepine (1000 mg orally once daily for ponies weighing 160 to 205 kg) or control supplement, beginning 5 days before being moved to a domestic facility for a 2-week introduction to stabling, haltering, leading, tethering, social separation, stall confinement, grooming, simulated girthing, lifting feet, health care treatments, and transportation. Objective quantitative behavior measures (latencies to complete tasks, avoidance responses, and nervous defecations) were derived from video-recorded handling sessions. For each of the 14 sessions, ponies were ranked 1 (best) to 6 (poorest) for calm, compliance, and acclimation/skill progress. All human–animal interactions, video analyses, and rankings were done blindly to supplement assignments. For most daily sessions across the 2-week training period, each of the three alpha-casozepine-treated ponies performed better than their matched control counterparts, and they also had the top three sums of daily session ranks, with a mean of 35.2 compared with 62.8 for control ponies ($P < .05$, dependent t-test). At 6 weeks after the 2-week training period, the alpha-casozepine-treated ponies retained the best three sum of ranks for the seven specific skills re-assessed at that time. These results provide evidence of the benefit of alpha-casozepine supplementation to horses undergoing potentially stressful situations inherent to domestic management.

1. Introduction

The nutritional supplement alpha-casozepine, a decapeptide derived from bovine milk α-$_{S1}$ casein, has been found to have calmative anxiolytic-like properties in humans [1-5] as well as several animal species, and in a variety of stress models. In rats, alpha-casozepine was found to have behavioral effects comparable with those of the anxiolytic diazepam, both for the conditioned defensive burying and the elevated plus-maze models of stressful events [6,7]. In cats, in a randomized blind clinical trial across multiple practices, treatment with oral alpha-casozepine resulted in significantly greater improvement in their interaction with humans compared with placebo, including fear of strangers, contact with familiars, general fears, and fear-related aggression [8]. In dogs, in similar

Corresponding author at: Sue M. McDonnell, PhD, Havemeyer Equine Behavior Lab, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, 382 West Street Road, Kennett Square, PA 19348.
E-mail address: suemcd@vet.upenn.edu (S.M. McDonnell).

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randomized blind clinical trials, treatment of anxiety-related disorders using alpha-casozepine resulted in improvement in rating on a standard inventory of emotional disorder symptoms similar to that obtained with the standard reference treatment with selegiline [9].

Domestic horses are particularly vulnerable to stress, often related to fear, during everyday handling and training, resulting in behavioral (eg avoidance, aggression, stereotypies) and physiologic (eg gastric ulcers, injury) responses impacting animal health and welfare as well as horse and human safety. Common potentially stressful experiences for horses include weaning, training for routine ground handling or performance, social regrouping or isolation, transportation, changes in housing and environment, as well as mildly aversive routine health care examination and treatment procedures and restraint, including dental and hoof care. Although appropriate pharmaceuticals are available and are practical for use in some circumstances, a nutritional supplement with anxiolytic-like calming effects would be more practical in many instances as an aid in reducing stress and its consequences.

The purpose of the current study was to evaluate the effects of alpha-casozepine on behavior and training efficiency of horses during potentially stressful experiences. The model used was acclimation of adult semi-feral ponies to domestic management, including movement from life-long family groups to new smaller enclosures and indoor environments, social separation and isolation, as well as grooming, haltering and leading, routine health care procedures, and transportation. Our assumption in this model is that more efficient acclimation/training progress with fewer stress and avoidance behaviors (such as stress vocalizations, nervous defecations, tail swishes, freezing, avoidance responses, or aggressive postures and responses to handling) reflects less fear or anxiety.

2. Methods

2.1. General Design

Six ponies, consisting of three matched pairs, with one of each pair randomly assigned to alpha-casozepine treatment and the other to control supplementation, were evaluated during standard procedures used routinely in this laboratory to transition previously untrained semi-feral animals to domestic housing, feeding, interaction with humans for basic ground handling, transportation, routine health care, and veterinary procedures in a stable setting. Handling was conducted over a 2-week period using positive reinforcement-based behavior modification protocols. Fear-response behavior, training progress, and retention of learned skills of alpha-casozepine-treated ponies and control ponies were compared. All animal handling, behavior assessments, data entry, and group comparisons were done blindly to group assignments.

2.2. Subjects

The subjects included six small Shetland-type (160-205 kg) ponies that had been born and kept continuously since birth under semi-feral herd social and environmental conditions. These included three pairs matched for gender, age, and known temperament. Pair 1 was a 2-year-old filly and her 3-year-old full sister; pair 2 included two 2-year-old colts; and pair 3 included two mature stallions, aged 6 and 8 years. The ponies were relatively naïve to interaction with humans compared with domestically reared adult horses. In their semi-feral management, the limited interactions had been based on all-positive reinforcement behavior-modification techniques similar to what was used in this study, where the goal is to minimize anxiety and fear. None of the ponies had shown indication of acquired aversions to interaction with humans in general or to any of the specific procedures to which they had been exposed during their previous semi-feral management (observation, height and weight tape measure estimation, blood sampling two to four times per year, annual vaccination, occasional oral deworming, testicular palpation and measure).

Animal procedures were approved by the University of Pennsylvania Animal Care and Use Committee.

2.3. Treatments

Alpha-casozepine supplement consisted of one standard, commercially prepared, daily equine dose of 1000 mg alpha-casozepine (Zylkene®, ORSCO, 14 Porte du Grand Lyon 01700 Neyron, France) mixed with ¼ cup Equine Senior pellets (Purina Mills, LLC, Gray Summit, MO, USA) for horses up to 500-kg body weight, hand-fed daily at 07:00 hour starting 5 days before separation from family bands and continuing through the subsequent 11 days of acclimation/training procedures. Control treatment consisted of a similar volume of oat flour ground to a visual consistency similar to that of the alpha-casozepine mixed with Equine Senior and fed on a similar schedule.

2.4. Management and Handling Procedures and Associated Behavior Measures

Acclimation/training sessions were conducted once or twice daily Monday through Friday over a period of 2 weeks for a total of 14 sessions. All handling procedures and acclimation to stabling were video recorded for subsequent review to derive quantitative measures of compliance and comfort with the procedures. Table 1 summarizes the handling and management schedule, with details of the procedures and associated objective measures recorded for analysis. All animal care and handling was done by two handlers experienced with the specific procedures and with acclimation/basic ground handling of semi-feral ponies in transition to domestic management. One handler served as the primary handler, whereas the other was available for handling assistance as judged necessary by the primary handler to avoid prolonged delay or negative experience.

In addition to the measures listed in Table 1, at the completion of each training procedure session and at the end of the training period, the two handlers independently and subjectively ranked the ponies based on clinical impressions of each animal's level of comfort (vs. stress) with the experience and overall ease and progress of training or acclimation. Also, at the end of each week of training, the two handlers independently ranked the ponies specifically for learning efficiency based on the rate

of simple associative learning for specific new skills and acclimation procedures.

Six weeks after completion of the 2-week training period, with no interim handling, retention of seven specific skills introduced during the acclimation/training period was assessed. Handling for this assessment was done by a third trainer skilled with the all-positive behavior-modification techniques, but with no previous interactions with these ponies or knowledge of the nature of the study or treatment assignments.

2.5. Data Analysis

For each handling session, considering all objective measures, the ponies were ranked from 1 (best) to 6 (poorest) for calm, compliance, and training/acclimation progress. In addition, for each subject, the ranks for 14 sessions were summed to derive an overall 2-week program score for statistical comparison of groups using dependent *t*-test procedures [10]. For the retention assessment, based on quantitative measures, ponies were ranked for each of the seven skills indicated in Table 1, and an overall retention score based on the sums of those ranks was derived. The handler also subjectively similarly ranked the ponies from 1 (best) to 6 (poorest) for performance on each procedure and overall for the seven skills.

3. Results

3.1. Rankings Based on Objective Measures

For 13 of 14 sessions over the 2-week training period, the average rank among the six ponies (derived from daily objective quantitative measures reflecting training progress and comfort) was better (1 = best of the 6 ponies, 6 = poorest) for alpha-casozepine-treated ponies as compared with that for control ponies. For 7 of 14 training sessions, each of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts. For 4 of the remaining 7 sessions, two of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts.

The final 2-week program scores (sum of 14 session ranks) were 35, 44.5, and 26 for the three alpha-casozepine-treated ponies, with a mean of 35.2 compared with 57.5, 63, and 68, respectively, for corresponding matched control ponies with a mean of 62.8 (lower sums represent better performance).The difference is significant ($P < .05$, dependent *t*-test, 2df).

For the 6-week assessment of retention of seven specific skills, three of the three alpha-casozepine-treated ponies and two of the three control ponies performed at or above levels reached during the 2-week training. For four of the seven specific skills compared (approach and catch at pasture, leading, lifting/picking out feet, intranasal application), as well as for overall performance, each of the three alpha-casozepine-treated ponies scored better than their matched control counterparts. For two of the remaining three specific skills compared (clipper and cross-tie), two of the three alpha-casozepine-treated ponies ranked better than their matched control counterparts.

3.2. Handlers' Subjective Rankings

The two handlers' subjective daily training session rankings of level of comfort (vs. stress) with the experience and overall ease and progress of training or acclimation that were independently recorded after each handling session were consistently in agreement with each other as well as with the rankings derived from objective quantitative measures. Similarly, the two handlers' independently recorded rankings of ponies' learning efficiency consistently placed the three alpha-casozepine-treated ponies as the best three learners. Also, for the retention assessment, the handlers placed the three alpha-casozepine-treated ponies as best overall for comfort, compliance, and skill level.

4. Discussion

In this model of transition of adult ponies from life-long semi-feral social and environmental conditions to domestic management and introduction to basic ground handling using primarily positive reinforcement-based methods, all three ponies fed alpha-casozepine performed better overall as compared with their matched control counterparts. These results provide evidence that the nutritional supplement alpha-casozepine holds promise as a valuable aid to improving efficiency of handling and training horses. The procedures introduced to these ponies represent a reasonable sample of the various ground handling and management experiences of domestic horses associated with fear- and stress-related behavior. Consistently more compliant behavior and greater learning efficiency across the sample of handling experiences common to domestic horses suggest that alpha-casozepine supplementation may be of benefit generally to horses in potentially stressful situations. Further investigation is warranted.

Although nonconfrontational positive reinforcement-based behavior modification methods of animal handling and training are gradually gaining favor, traditional handling styles based on negative reinforcement and punishment likely remain more common throughout the equine industry. Although we would advocate more widespread use of positive reinforcement-based handling of horses, further work should address effectiveness of alpha-casozepine with different handling styles.

In this study, daily treatment commenced 5 days before the ponies were moved from semi-feral to domestic management and continued through the 2 weeks of acclimation and training. Alpha-casozepine has recently been marketed in Europe as a feed supplement for horses and ponies; it is available in 1000-mg dose packets, with the label instruction to feed one packet daily per horse or pony weighing up to 500 kg, and two packets for horses weighing more than 500 kg, starting 2 days before anticipated stressful experience. Daily milligram/kilogram dose would vary considerably across the range of body weights. These ponies, weighing 160 to 201 kg, were fed approximately 4.9 to 6.2 mg/kg daily. In clinical trials with cats [8], dogs [9], and humans [1-4], once daily oral dosing was found effective. In rat experiments, oral dosing 1 hour before the test procedures was also found effective [7]. Further work is needed to investigate effective dose rates and regimens for horses and ponies, particularly exploring

Table 1
Daily acclimation/training sessions and specific objective measures

Session	Procedures	Measures
Week 1		
Day 1	Separation from family bands, loading into stock trailer, 1-mile transport followed by unloading into paddock with run-in shelter together with other subjects	Defecations (number and consistency), assistance required loading, latency to load (minute), assistance required unloading
Day 2	Catch and halter at pasture, lead into training barn for first experiences with multiple types of man-made surfaces (wet and dry), enclosed space with ceilings, walls, artificial lighting, rubber mats, metal floor drain grates, windows, shadows, barn furnishings. Introduction to grooming, introduction to tethering and cross-ties as judged ready	Latency to approach and halter in paddock, hesitations or stops during leading, latency to enter barn (minute), leading assists requested, defecations, percent time tolerating grooming, avoidance behaviors (pull back on lead, rear, spin, stamp, kick, bite, lunge forward)
Day 3 (a)	Repeat of day 2 leading and grooming plus start of training for foot lifting and acclimation to electric clipper	As day 2, plus foot lifting attempts and successes, percentage of time tolerating clipper and progress with clipper touch to body
Day 3 (b)	Obstacle course 2 m wide by 9 m long with wet concrete footing, stainless lab cart, empty feed bag on floor, floor drain grate running through the center of the course, video tripod, clear plastic bag on floor, brown paper bag hanging from ceiling to pony height moving in breeze, a 0.6-m high red plastic trash bin, 2 m × 3 m green fabric tarp on floor, and 0.6 m × 0.6 m bright blue wobbly metal floor plate	Latency (seconds) to complete obstacle course, hesitations, stops, leading assists requested, defecations, vocalizations
Day 4	Obstacle course as day 2 with different obstacles expected to present greater challenge, including a black hose on the floor dripping condensation, a water sprinkler fountain with run-off stream and puddle, the floor drain grate median, a snow shovel on floor, three black rubber feeding pans mounted on a board on the floor, a 20-L insulated water cooler, a hanging blinker hood, and the blue wobbly metal floor plate as above	As above
Day 5	Initial social isolation and stabling in a box stall (three solid walls with open grill front) within the training barn for 1.5 hours, with no other animals in the barn	Defecations, latency to become calm enough to eat hay, pawing bouts, vocalizations, escape attempts (climb, push, lunge at walls or stall front doors)
Week 2		
Day 6 (a)	Obstacle course in new 7 m × 7 m room brightly lit with both incandescent and fluorescent, with cluttered set of new obstacles: up steep ramp entry with textured rubber flooring, through narrow examination chute with hanging tarp curtain, AM radio with static, camera and monitor on cart, open door with breeze blowing curtain and 0.6-m square plastic bag on floor, wheeled muck cart, metal step ladder, tripod, 0.6 m × 6 m mirror, stool with tote box and sprinkler (not running) on top, leather dummy mount, hanging red fabric bag, red plastic trash bin 0.6-m high, followed by continued handling acclimation as above at the cross-ties in the following standard order: rectal temperature, height and weight tape measurement, sweep broom near pony on crossties, brush all over, drop plastic shovel to floor nearby, run clippers on body, saddle pad and girth, walk a 30-m distance with saddle pad and girth	Latency to complete obstacle course (minute), foot lifting attempts and successes, percentage time tolerating grooming, percentage time tolerating clipper and progress with clipper, defecations, avoidance behaviors as above
Day 6 (b)	Continued stall acclimation with six ponies in individual adjacent stalls together in the same barn for 2.5 hours	As on day 5 stall acclimation
Day 7	15-Minute battery of management, health care and veterinary procedures at cross-ties: jugular stick 1, IM needle stick, simulated intranasal vaccination, oral dewormer, spray bottle misting of body and legs, cold water hosing of body and legs, jugular stick 2	Latencies to complete each procedure, ease of introduction to spray misting, ease of introduction to hosing, avoidance behaviors as above
Day 8	Leading assessment over standard ¼ mile course over new terrain and in new buildings including through water, over hard and soft surfaces, new indoor spaces, variable natural and artificial lighting, narrow chutes, a hollow sounding wood floor, and near pastured groups of horses	Latency to complete ¼ mile course, nervous tail swishes, hesitations, stops, vocalizations, defecation, avoidance behaviors as above
Day 9 (a)	Load and stand in examination stocks for 10-minute veterinary examination per rectum with U/S imaging	Latency to load into stocks (second), avoidance behaviors as above, latency to relaxation during per rectum examination
Day 9 (b)	Leading across series of rubber floor mats of three different colors followed by introduction to ear clipping at cross-ties	Latency to be led cross series of red, white, and black rubber floor mats, hesitations, stops; latency (minute) to tolerate clippers applied to the poll and ears, avoidance behaviors as above

Day 10 (a)	Feet lifting assessment, holding each foot up for ≥ 3 seconds	Latency (minute) to complete all 4 feet, avoidance behaviors as above
Day 10 (b)	Loading into stock trailer as on Day 1 for 2.6-mile transport alone followed by unloading	Latency to load, defecations, vocalizations, avoidance behaviors as above, latency to unload
Assessment of skill retention at 6 weeks		
	Catch and halter at pasture, lead ¼ mile previously untraveled route past multiple social challenges to cross-ties in training facility, lift and briefly pick out 4 feet, apply clipper to body, poll, and ears, simulated intranasal vaccination, hosing of legs and body and stand at cross-ties unattended for 5 minutes, and return ¼ mile route, all with a novel handler	Latency to approach and halter at pasture, latency to complete novel route, hesitations, stops, defecations, assists needed, latencies and avoidance responses and defecations to lift and pick feet, apply clipper, complete intranasal treatment, hosing of legs and body, and stand unattended at cross-ties, and return leading ¼ mile

acute treatment as reported for rats, that would be practical in many instances where a problematic aversion develops amidst the need to complete a procedure in a timely fashion, for example during veterinary care.

Alpha-casozepine and other milk proteins and metabolites with demonstrated anxiolytic-like effects are currently believed to work though the GABA neurotransmitter system, the same system mediating effects of the benzodiazepine anxiolytic agents [11,12]. The benzodiazepines were among the first generation of tranquilizers with anxiolytic effects at dose levels that did not impair physical coordination and mental alertness. Nonetheless, benzodiazepines are known for certain adverse effects on memory as well as reduced threshold (or disinhibition) for aggression. The benzodiazepine diazepam has been used as an anxiolytic in horses, within the specific context of releasing inhibited sexual behavior in stallions [13-15]. In this context in stallions, simultaneous disinhibition of aggressive behavior is a common undesirable side effect, which often becomes counterproductive if not anticipated by skillful handlers or tolerated by the stimulus mare. This conspicuous disinhibition of aggression in stallions, along with purported adverse effects on memory in other species, is likely the main reason that this class of calmative agents has not been explored for more general application in horses. A promising feature of the milk protein-derivative supplements is that, although apparently GABA-mediated, their effects appear to be specific to anxiety, without the adverse effects on memory or disinhibition of aggression typical of benzodiazepine anxiolytics [11,12]. The alpha-casozepine-treated ponies in this study appeared normally alert. Learning ability, based on acclimation and training efficiency across a broad range of experiences, was greater for the alpha-casozepine-treated ponies. Learning progressed from day-to-day during the training/acclimation period, and skill and acclimation were well retained when assessed at 6 weeks. Similarly, although there was a generally low level of aggression elicited in this nonconfrontational handling method, there was no indication of disinhibition of aggression in the alpha-casozepine-treated ponies. Because styles of horsemanship vary within the equine industry such that many horses are handled and trained in a more confrontational style using principally negative reinforcement and punishment that tends to inadvertently elicit dangerously fear-responsive reactions that threaten animal welfare and human safety, further study specifically addressing effects of milk protein-derived calmatives on aggression is warranted.

In conclusion, the results of this blindly conducted study indicate the benefit of alpha-casozepine dietary supplement to ponies undergoing transition from semi-feral management to domestic management and handling. These findings in horses support a growing body of research and clinical evidence for a calmative anxiolytic-like effect of alpha-casozepine in mammals.

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Health. The study was designed and conducted and the manuscript written by Dr. McDonnell in consultation with Dr. Vaala and with the technical assistance of University of Pennsylvania employees Ms. Jaime Miller and Mr. Andrew Debenham.

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alpha-Casozepine

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Abstract

We found previously that dipeptide YL exhibits orally active anxiolytic activity comparable to diazepam. The YL sequence is often observed in the primary structure of natural food proteins. In the present study, we investigated whether YL and YL analogues are released from bovine αS-casein by gastrointestinal proteases. YLG, corresponding to αS1-casein (aa 91-93), was more effectively released from αS-casein than YL by pepsin-pancreatin digestion, mimicking gastrointestinal enzymatic conditions. Using the synthetic model peptide, we determined that trypsin cleaved the N terminus of YLG, and elastase and carboxypeptidase contributed to cleave the C-terminus. YLG exhibited orally active anxiolytic-like activity in the elevated plus maze and open-field tests in mice. The anxiolytic-like activity of YLG was inhibited by WAY100135, SCH23390 or bicuculline, antagonists of serotonin 5-HT1A, dopamine D1, and GABA(A) receptors, respectively; however, YLG had no affinity for these receptors. The pepsin-pancreatin digest of αS-Casein also exhibited anxiolytic-like activity. Meanwhile, anxiolytic-like activity of α-casozepine, an αS1-casein-derived decapeptide with YL sequence in the N terminus, was blocked by WAY100135, SCH23390, or bicuculline, equally to YLG and YL; however, it was not detected in the pepsin-pancreatic digest. Taken together, we found that YLG is released after pepsin-pancreatic digestion of αS-casein and exhibits potent anxiolytic-like activity via activation of serotonin, dopamine, and the GABA receptor system.

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Characterization of α-casozepine, a tryptic peptide from bovine α_{s1}-casein with benzodiazepine-like activity

Laurent Miclo*, Emmanuel Perrin*, Alain Driou*, Vassilios Papadopoulos[†], Noureddine Boujrad[†,#], Régis Vanderesse[‡], Jean-François Boudier[§], Didier Desor[‖], Guy Linden*, and Jean-Luc Gaillard*

*Laboratoire des Biosciences de l'Aliment UA 885 INRA, Faculté des Sciences, Université Henri Poincaré-Nancy 1, Vandoeuvre-lès-Nancy, France; [†]Departments of Cell Biology, Pharmacology and Neuroscience, Georgetown University Medical Center, Washington, DC; [‡]Laboratoire de Chimie Physique Macromoléculaire UMR 7568 CNRS, ENSIC, INPL, Nancy, France; [§]Ingredia, Arras, France; [‖]Laboratoire des Aspects Fonctionnels et du Développement des Comportements URA 1293 CNRS, Faculté des Sciences, Université Henri Poincaré-Nancy 1, Vandoeuvre-lès-Nancy, France; and [#]Endocrinologie Moléculaire de la Reproduction UMR 6026 CNRS Interactions Cellulaires et Moléculaires, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes Cedex

Corresponding author: Laurent Miclo, LBSA, Faculté des Sciences, Université Henri Poincaré, B.P. 239, 54506 Vandœuvre-lès-Nancy, France. E-mail: Laurent.Miclo@scbiol.uhp-nancy.fr

ABSTRACT

Caseins are a known source of biologically active peptides. In this study, we have shown evidence of a novel anxiolytic activity in a tryptic hydrolysate of bovine α_{s1}-casein. Injection of 3 mg/kg of this hydrolysate significantly reduced the epileptic symptoms caused by pentylenetetrazole in rats. Anxiety reduction was also observed when the hydrolysate was tested in the elevated plus-maze and in the conditioned defensive burying rat models. Peptides isolated from the hydrolysate were examined for their affinity for the γ-amino-butyric acid (GABA) type A receptor. Only one peptide, named α-casozepine, corresponding to the 91–100 fragment from bovine α_{s1}-casein, expressed affinity for $GABA_A$ receptor. *In vitro*, the peptide had 10,000 less affinity for the benzodiazepine site of the $GABA_A$ than did diazepam. However, in the conditioned defensive burying paradigm it was 10-fold more efficient than diazepam. The difference observed between the *in vitro* and *in vivo* activity of α-casozepine could not been explained by an action via the peripheral-type benzodiazepine receptor; α-casozepine had no affinity for this receptor. The α-casozepine amino acid sequence could be related to the carboxy-terminal sequence of the polypeptide diazepam binding inhibitor, an endogenous ligand of the central $GABA_A$ and peripheral-type benzodiazepine receptors.

Keywords: milk • casein peptide • anxiolysis • anticonvulsant • diazepam binding inhibitor

Many physiological benefits are attributed to milk. A gastroprotective action in an *in vivo* rat stress-induced ulcer model of numerous dairy foods, including cream, whole milk, and skim milk, has been reported (1). In folk wisdom, milk intake would improve sleep

or provide a calming effect. More than 60 years ago, it was reported that adults showed a higher tendency toward uninterrupted sleep after consuming a meal of cornflakes and milk (2). Brezinova and Oswald (3), using electroencephalography, have found that sleep of old people is significantly longer and less broken after cereal–milk intake at bedtime and that the action of milk is more effective with serial administration. Those results would be associated with a conditioned response to milk that persists from infancy.

Milk proteins are the only proteins synthesized by mammals in order to feed their young. The effects of caseins in addition to being nitrogen providers for the newborn are considered, because many works have shown in the past 15 years that their enzymatic hydrolysis produces peptides with various biological activities (4). The peptides found are opioid and opioid-antagonist peptides, angiotensine-converting-enzyme inhibitors, immunostimulating peptides, platelet-aggregation inhibitors, phosphopeptides carriers of minerals (Ca^{2+}, Fe^{2+}), mitogenic peptides, antibacterial peptides, and protease inhibitors. Their physiological role is often putative as most of these properties have been discovered by *in vitro* experiments. However, opioid peptides have been detected *in vivo* in duodenum of mini-pigs after bovine milk intake (5), in human adult gut after milk ingestion (6), in serum of newborn calves after first milk intake (7), and in plasma of pregnant or breast-feeding women (8). *In vivo* activities have been evidenced for angiotensine-converting-enzyme peptidic inhibitors, because per os ingestion of fermented milk containing these peptides reduces blood pressure in rats suffering from genetic hypertension (9). Nevertheless, the physiological purposes of the biological active peptides from milk caseins are not yet elucidated (10).

These considerations have led us to try to determine whether the popular sedative and calming properties of milk could be carried by a peptide. On the one hand, the compounds reducing the function of the GABA-coupled chloride channel, as beta-carboline derivatives, produce pharmacological effects such as anxiety and convulsions (11, 12). On the other hand, benzodiazepines (BDZs) or barbiturates, which enhance the GABAergic transmission, are anxiolytic and anticonvulsant. So, the $GABA_A$ receptor complex plays a major role in the pharmacology, neurochemistry and physiopathology of stress and anxiety (13, 14). A deficiency of GABAergic transmission is also linked to epilepsy, because the inhibition of the expression of the $GABA_A$ receptor $\gamma 2$ subunit by antisense oligonucleotide causes electrographic seizures (15). A second BDZ-binding site with a predominantly mitochondrial localization has been identified and named “peripheral-type benzodiazepine binding site” (PBR), although it is present in all tissues, including the central nervous system.

PBRs are implicated in steroidogenesis (16). Drugs that bind to PBR in the brain may regulate neurosteroid production by glial cells, which in turn can act on the neuronal $GABA_A$ receptor and modulate neuronal activity and brain function (17, 18). Some BDZs as 4’-chlorodiazepam (Ro5-4864) bind with high affinity to the PBR and isoquinolines, which are not BDZs, have a great selectivity for this receptor.

In the present study, we show that tryptic hydrolysate of bovine α_{s1}-casein have *in vivo* BDZ-like activity. We identified the active peptide by *in vitro* screening by using the γ-amino butyric acid type A ($GABA_A$) receptor binding assay and was checked for *in vivo* activity.

MATERIALS AND METHODS

Bovine α_{s1}-casein preparation

Raw bovine milk from Holstein cows was skimmed by centrifugation (2,000 *g*, 20 min, 30˚C). Caseins were precipitated by adjusting milk to pH 4.6 with 1 M HCl. After centrifugation (1,500 *g*, 20 min, room temperature), the precipitate was washed twice with distillated water and solubilized at pH 7.0 with 1 M NaOH. The precipitation at pH 4.6, washing steps and resolubilization in water at neutral pH were repeated twice. Sodium caseinate was lyophilized after the last solubilization at pH 7.5 with 1 M NaOH. α_{s1}-Casein was prepared from sodium caseinate by batch fractionation adapted from that described in ref. 19. A quantity of 20 g of dry diethylaminoethyl (DEAE)-cellulose DE23 (Whatman, Maidstone, UK) was equilibrated in 150 ml of a 0.02 M sodium acetate buffer, pH 6.6, with 3.3 M urea, 0.035 M EDTA, and 0.1% (v/v) 2-mercaptoethanol and was mixed with 5 g of sodium caseinate solubilized in 100 ml of the same buffer. DEAE-cellulose and sodium caseinate were mixed gently for 15 min and filtered through Whatman n°41 filter paper. The DEAE-cellulose cake was dispersed in 250 ml of the same buffer, and the procedure was repeated twice. Elution of caseins was performed in two steps: first with 35 mM $CaCl_2$ and then with 70 mM $CaCl_2$ dissolved in 0.02 M sodium acetate buffer with 3.3 M urea. Each elution step was repeated twice. Filtrates were dialyzed and lyophilized.

Tryptic hydrolysis of α_{s1}-casein

α_{s1}-Casein was dissolved in 50 mM ammonium formate buffer pH 8.5 to a final concentration of 0.2% (w/v). Seven N-α-benzoyl-arginine-ethyl-ester units of immobilized trypsin from bovine pancreas (EC 3.4.21.4) (Sigma, St. Louis, MO) were added to the solution, and hydrolysis was performed during 1 h at 37˚C with gentle stirring. Hydrolysis was stopped by centrifugation (1,700 *g*, 5 min, 4˚C). The supernatant was evaporated under vacuum to remove volatile buffer and α_{s1}-casein total hydrolysate was redissolved in distilled water. The evaporating procedure was repeated five times. α_{s1}-Casein hydrolysate was then lyophilized.

Purification of α_{s1}-casein tryptic peptides

α_{s1}-Casein peptides were resolved by reversed-phase chromatography by using a Hitachi-Merck system with an L6200 ternary pumping system, a Model 655A-40 automated injection and a sampling system (Merck, Darmstadt, Germany) coupled with a Waters Model 996 photodiode array detector controlled by a computer. Casein hydrolysate was run on a LichroCart C_{18} column (250 × 4 mm i.d., 5 µm particle size, 10 nm porosity) (Merck). Peptides were eluted with a gradient from 5% to 40% of acetonitrile (Rathburn, Walkerburn, UK) in Ultra-High Quality water containing 0.1% (v/v) trifluoroacetic acid (TFA) (sequencing grade, Sigma) for 70 min at a flow rate of 1 ml/min. Peptides were collected manually. After lyophilization, these partially purified peptides were re-run in isocratic conditions. Peptidic fractions were lyophilized.

Amino acid analysis

Purified peptides were hydrolyzed in 6 M HCl with 0.5% (w/v) phenol and 0.1% (v/v) 2-mercaptoethanol under vacuum at 110 ± 2˚C during 24 h. The amino acid compositions were

performed on a cation exchanger resin BTC 2410 with a Biotronik LC3000 analyzer (Munich, Germany) by using a ninhydrin derivatization of amino acids.

Peptide sequence

The sequence was determined in an automatic Edman degradation microsequencer Model 476A (Perkin Elmer Applied Biosystems Division, Foster City, CA).

Mass analysis

Mass of α_{s1}-casein peptides was checked by fast atom bombardment-MS (FAB-MS). FAB-mass spectra were acquired on a VG ZAB-HF double-focusing mass spectrometer (VG Instruments, Manchester, UK). The matrix used was thioglycerol, and samples were dissolved in methanol for loading onto the target. Ionization was achieved with a fast atom gun operated at 10 kV. For peptides in which mass was superior to 3,000 Da, we performed analysis by electrospray ionization-MS (ESI-MS) on a Bio-Q quadrupole mass spectrometer (VG Instruments) equipped with an electrospray ion source operating in the positive-ion mode. Peptidic fraction was mixed with 50% acetonitrile and 1% formic acid in water before positive ion electrospray. Scanning was from m/z 500 to 1500. Bovine α_{s1}-casein was analyzed by ESI-MS in the negative-ion mode.

Peptide synthesis

The peptide was synthesized on a Dupont Coupler 250 peptide synthesizer (DuPont Co., Wilmington, DE) from Nα-tert-butoxycarbonyl(Boc)-mesitylene-2-sulfonyl-arginine-4-(hydroxymethyl)-phenylacetamidomethyl-polystyrene resin (0.56 mmol/g substitution, Neosystem, Strasbourg, France), by using Boc solid-phase peptide-synthesis protocols. The peptide chain was assembled starting from 0.5 mmol mesitylene-2-sulfonyl-arginine linked to the resin by 2 h coupling of the Boc-Xaa (1.5 mmol), with the side chains of glutamic and tyrosine residues protected, respectively, by benzyl ester and 2,6-dichlorobenzyl ester in the presence of 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium tetrafluoroborate (1.5 mmol), and diisopropylethylamine (4.5 mmol) in dimethylformamide/dichloromethane (1:3). The completion of each coupling step was monitored by Kaiser ninhydrin and 2,4,6-trinitrobenzenesulfonic acid tests. Deprotection of the Boc group was achieved by 40% (v/v) TFA in dichloromethane. A standard cleavage with 18% (v/v) trimethylsilyl trifluoromethane sulfonate, 65% (v/v) TFA, 11% (v/v) thioanisol, and 6% (v/v) 1, 2-ethanedithiol afforded the crude peptide. Desalting was achieved on Sephadex G-25 with n-butanol/pyridine/acetic acid/water (15:10:3:12) as eluent. Peptide was purified by HLPC on a LichroCart C_{18} column as described above.

$GABA_A$ receptor binding assay

Binding of tryptic α_{s1}-casein hydrolysate and purified peptides to $GABA_A$ receptors was studied by the drug discovery system kit (Nenquest™, NEN Life Sciences, Boston, MA) consisting of bovine cerebral cortex membranes in sodium phosphate buffer pH 7.7, [methyl-^{3}H]flunitrazepam (specific activity 83 Ci/mmol), and 2.5 μM flunitrazepam in 0.05 M Tris-HCl buffer pH 7.7 (binding buffer). For the binding assay, 400 μl of the membrane solution was incubated at 4˚C for 60 min with 50 μl [methyl-^{3}H]flunitrazepam (0.48 nM final concentration) and either 50 μl of

binding buffer to determine total binding or 50 µl of binding buffer containing nonradioactive flunitrazepam (0.25 µM final concentration) to determine nonspecific binding, or 50 µl of binding buffer containing various concentration of unlabeled flunitrazepam (final concentration ranging from 12.5 nM to 0.39 nM) to establish a standard curve, or peptides or peptidic fractions to be tested in 50 µl of binding buffer (final concentration ranging from 10^{-4} to 10^{-7} M). Ice-cold binding buffer (3 ml) was added, and rapid filtration through Whatman GF/B filter under vacuum stopped the incubation. Filters were washed twice with 3 ml of the same ice-cold buffer and put in counting vials with 10 ml of scintillation fluid (Ultima Gold, Packard, Meriden, CT). After one night at room temperature, the radioactivity was measured during 10 min on a Betamatic V counter (Kontron Instrument, Milton Keynes, UK s). Nonspecific binding value was subtracted. IC_{50} and Hill number were obtained from Scatchard plot representation.

PBR radioligand binding assays

The assays were performed on MA-10 mouse Leydig cell mitochondrial preparations. MA-10 cells contain high levels of PBR localized primarily on the mitochondria (17). MA-10 cells were scraped from 150 mm culture dishes into PBS and centrifuged at 500 *g* for 15 min. Mitochondria were prepared by differential centrifugation as previously described (20) and resuspended in PBS at 1 µg to 10 µg of protein per sample. [N-methyl-^{3}H]Ro5-4864 (specific activity 86 Ci/mmol) and [N-methyl-^{3}H]PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide; specific activity 75 Ci/mmol), were obtained from NEN. PK 11195 and Ro5-4864 were obtained from Research Biochemicals Incorporated. [^{3}H]PK 11195 or [^{3}H]Ro5-4864 binding studies were performed at 4˚C, in a final incubation volume of 0.3 ml, by using radioligands in the concentration range of 0.03–20 nM and 200-fold excess of unlabelled ligand, as previously described (17). Competition studies were carried out with a concentration of radioligand from 1 nM to 5 nM, and increasing amounts of the peptide. After 90 min incubation, assays were stopped by filtration through Whatman GF/B filters equilibrated in 0.1% polyethyleneimine and washed with 20 ml ice-cold PBS. Radioactivity trapped on the filters was determined by liquid scintillation counting. The dissociation constant (Kd) and the number of binding sites (B_{max}) were determined by Scatchard plot analysis of the saturation isotherms generated by using the LIGAND program (KELL, v.4.0, Biosoft, Inc., Ferguson, MO) (21). The IC_{50} from competition studies were also calculated by using the LIGAND program.

Sequence alignment

Alignment of diazepam binding inhibitor (DBI) from various species with α_{s1}-CN-(f91-100) was performed according to CLUSTALW method (22).

Subjects

A group of 60 non-blood-related Wistar rats, weighing 180 to 230 g at the beginning of the elevated plus-maze experiment; 27 rats weighing 230 to 260 g at the beginning of the anticonvulsant experiment; and 48 rats weighing 280 to 300 g at the beginning of the conditioned defensive burying experiment were obtained from Iffa Credo (Saint-Germain sur l'Arbresle, France). Animals were adjusted to the laboratory environment 3 weeks before the beginning of the experiment. On receipt, the rats were individually identified and housed by groups of four to avoid the isolation stress in an air-conditioned room maintained at a constant temperature (22 ±

2˚C) with 12 h inverted day/night cycle (light 8:00 p.m. to 8:00 a.m.). Tap water and standard diet (Extralabo, Pietrement, Provins, France) were available ad libitum. During the acclimatization period of two weeks, rats were weighed three times by week to avoid manipulation stress. Experiments were performed during the obscure phase of the cycle. All animal studies were conducted in accordance with the principles and procedures defined by the French Ministère de l'Agriculture et de la Pêche under agreement number A54540.

Elevated plus-maze experiment

Rats were randomly divided into three groups. The first group received an i.p. injection (2 ml/kg) of an aqueous solution of 0.5% (w/v) gelatin and 5% (w/v) mannitol. The second group received an i.p. injection of diazepam (2 mg/kg) (Hoffman-La Roche, Basel, Switzerland) suspended in the gelatin–mannitol solution. The last group received an i.p. injection of α_{s1}-casein hydrolysate (3 mg/kg) solubilized in the gelatin–mannitol solution. Rats were tested in the elevated plus-maze 30 min after the injection. They were removed from their cages and, according to Pellow et al. (23), placed in a box (30×30×30 cm) 5 min before the beginning of the experiment. Pellow et al. (23) describe the plus-maze in detail. The maze consisted of a cross with two opposite open arms and two opposite enclosed arms with 40 cm high walls. The apparatus was elevated at 50 cm from the floor. Rats were placed individually in the center of the maze, which was cleaned after each rat in order to avoid persistent smells, and their behavior tape-recorded for 5 min. The videotape observer was unaware of the experimental conditions of the animals. The number of entries into open and enclosed arms and time spent on open arms were scored. The percentage of entries in open arms was also calculated. The number of entries into closed arms provides a measure of general activity. Data were analyzed using ANOVA (24).

Inhibition of pentylenetetrazole-induced seizures

On day 1, each of the 27 rats received an i.p. injection of 25% (v/v) dimethyl isosorbide ether (DMI) (Sigma) solution in water 30 min before receiving an i.p. injection of pentylenetetrazole (PTZ) (Sigma) (60 mg/kg) in saline (control n°1). Only the 17 rats that had developed seizures were then used for the control and treatment experiments. Such a procedure decreases the variance because response to PTZ differs among rats. For each experiment, an individual rat was observed during the 45 min following the PTZ injection. The parameters scored: crisis severity according to Racine scale (25) (“0” means absence of crisis after PTZ administration, “5” means complete crisis with balance losing); crisis latency (time between PTZ injection and the first visible sign of crisis); and crisis duration (time between the first and the last visible sign of crisis). On day 4, the rats received an i.p. injection of α_{s1}-casein tryptic hydrolysate (1 mg/kg) dissolved in 25% (v/v) DMI in water 30 min before the i.p. injection of PTZ (60 mg/kg) (assay n°1). On day 6, rats received an i.p. injection of 25% (v/v) DMI in water 30 min before the i.p. injection of PTZ (60 mg/kg) (control n°2). On day 10, the same experiment as day 4 was performed with an increased α_{s1}-casein tryptic hydrolysate dose (3 mg/kg) (assay n°2). Finally, on day 12, a third control was done (control n°3) under the same conditions as on days 1 and 6. Data were corrected by using the transformation $x \rightarrow \log(1+x)$. Analyses were performed by using repeated measures ANOVA procedure corrected by Greenhouse–Geisser epsilon (26). Multiple comparisons were performed by using Fischer’s PLSD test. Assay n°1 was compared with control n°1 and assay n°2 to control n°2.

Conditioned defensive burying experiment

The conditioned defensive burying test is based on Pinel and Treit's procedure (27). On each of the 2 days before the test, the rats were placed individually in the test chamber without the shock-probe for 20 min. The conditioned defensive burying test was performed during the obscure phase of the cycle. The floor of the test chamber was covered with 5 cm of bedding material made of wood sawdust. On the center of one wall 2 cm above the level of the bedding material was a small hole through which the shock-probe was inserted. The rat was placed in the test chamber on the side opposite to the shock-probe. A single 2 mA shock was manually delivered when the rat touched the shock-probe with its forepaws for the first time. Once the rat received the shock, it recognized the shock-probe as the aversive stimulus. Burying behavior consists in a series of rapid and alternating movements of animal forepaws, moving and pushing a pile of bedding material over this aversive stimulus. The behavior of the rat was recorded for 5 min after it received the shock.

The 48 rats were put randomly into four groups. The first group received an i.p. injection (2 ml/kg) of a 0.9% (w/v) NaCl solution, the second group received an i.p. injection of diazepam (1 mg/kg) suspended in 0.9% NaCl solution, the third group received an i.p. injection of α_{s1}-casein hydrolysate (3 mg/kg) solubilized in 0.9% NaCl solution, and the last group received an i.p. injection of purified α_{s1}-CN-(f91-100) (0.4 mg/kg) solubilized in 0.9% NaCl solution. Compounds were injected 30 min before the test. An observer, who analyzed the results, was unaware of the experimental conditions of the animal. Duration of probe burying, number of head stretchings towards the probe, number of approaches towards the probe, and number of retreats away from the probe were scored. The percentage of approaches toward the probe followed by retreats was calculated (i.e., the ratio of the retreats to the approaches). An approach was scored when the animal approached the shock-probe at less than 2 cm or touched it. A retreat was scored when an animal, close to the shock-probe, ran away speedily at the opposite end of the probe. A subject that was not shocked after 5 min was discarded from the study. We analyzed all data by using ANOVA (24).

RESULTS

Preparation of α_{s1}-casein tryptic hydrolysate

The purity of α_{s1}-casein prepared by batch chromatography with $CaCl_2$ step gradient elution was estimated to be 96% by PAGE with 4 M urea. Analysis of α_{s1}-casein by ESI-MS shows most intense mass anion at m/z 23617. The B variant of bovine α_{s1}-casein-8P, which is the major variant in Holstein cows, has a calculated M_r of 23615. Mass anion m/z 23696 was also observed. The difference m/z 79 observed between the two mass anions corresponds to the molecular mass of the phosphate group of a phosphoseryl residue. Thus, the α_{s1}-casein fraction contained α_{s1}-casein-8P and α_{s1}-casein-9P, of which the sequence contained an additional phosphorylated serine residue. The main α_{s1}-casein tryptic peptides (Fig. 1) were characterized and identified by retention time and spectral analysis in reversed-phase HPLC (28), amino acid analysis and FAB-MS. In our conditions, we found nonspecific tryptic cleavage, whereas some bonds involving lysine or arginine residue were found totally or partially resistant to tryptic hydrolysis. The Arg^1-Pro^2 bond, which has been already described by Kaminogawa et al. (29),

and the Lys^{36}-Val^{37} bond were totally resistant to trypsin in our experimental conditions. The Lys^{105}-Val^{106}, Lys^{102}-Lys^{103}, Lys^{83}-Glu^{84}, Lys^{132}-Glu^{133} bonds were only partially cleaved by trypsin. After hydrolysis, the volatile salts were removed by evaporation under partial vacuum, and the lyophilized hydrolysate contained only 18 ± 5% (w/w) of ammonium formate.

α_{s1}-Casein tryptic hydrolysate anticonvulsant activity

Anticonvulsant activity of the hydrolysate was assessed *in vivo* with rats. A value of 2,700 s (observation duration) was arbitrarily assigned to crisis latency when an animal did not develop an epileptic crisis. Figure 2 shows a sensitization of animals to PTZ with increasing experiences. The latency of crisis significantly decreased from 156 ± 10 s (control n°1) to 116 ± 10 s (control n°3) ($P<0.01$). Difference was also significant between controls n°1 and n°2 (125 ± 12 s) ($P<0.02$). The same phenomenon was observed with the clonus duration because the mean value increased nonsignificantly about 4 min between control n°1 (562 ± 122 s) and control n°3 (801 ± 169 s). No modification in crisis severity was observed in the course of the three control experiments, because animals were selected according to their great sensitivity to PTZ. Intraperitoneal injection of 1 mg/kg of α_{s1}-casein tryptic hydrolysate significantly reduced the severity of the crisis ($P<0.02$). Compared with the first control, the mean value of the severity decreased from 3.7 ± 0.2 on Racine scale to 2.8 ± 0.4. With this dose, the clonus duration did not differ significantly from the control and the increase of crisis latency was not significant. The i.p. injection of 3 mg/kg of α_{s1}-casein tryptic hydrolysate led to a very significant reduction of PTZ action. Compared with control n°2, the mean value of crisis severity decreased from 3.6 ± 0.4 to 2.1 ± 0.5 ($P<0.002$) (Fig. 2A). The mean value of crisis latency significantly increased ($P<0.005$) from 125 ± 12 s to 1047 ± 314 s and the mean value of clonus duration significantly decreased from 728 ± 174 s to 404 ± 123 s ($P<0.005$). Because of the sensitization of animals during experiments, α_{s1}-casein tryptic hydrolysate would probably have a more intense effect. These results show a significant antagonist effect of α_{s1}-casein tryptic hydrolysate on PTZ action in Wistar rats.

α_{s1}-Casein tryptic hydrolysate anxiolytic activity

The elevated plus-maze test was used to evaluate the anxiolytic effect of the α_{s1}-casein hydrolysate (Fig. 3). The general activity of the rats on the plus-maze was not modified by saline, diazepam, or α_{s1}-casein tryptic hydrolysate i.p. injection because the number of closed-arm entries did not differ significantly among the three groups. The percentage of time spent in the open arms differed significantly in the three groups ($P<0.02$). The diazepam-treated rats spent significantly more time in the open arms than did the control rats. For the α_{s1}-casein tryptic hydrolysate, this parameter was not raised significantly. The percentage of time spent in the open arm is generally less straight than the percentage of entries in the open arms to measure anxiety (30). Diazepam and α_{s1}-casein tryptic hydrolysate significantly ($P<0.02$) enhanced the percentage of entries into the open arms (15.1 ± 3.0% of entries for the saline control group vs. 33.8 ± 5.9% and 29.3 ± 5.3% for the diazepam and α_{s1}-casein tryptic hydrolysate-treated groups, respectively). An experiment of conditioned defensive burying was carried out on male Wistar rats to confirm the anxiolytic activity of the α_{s1}-casein tryptic hydrolysate. Similarly to diazepam (1 mg/kg i.p.), the α_{s1}-casein tryptic hydrolysate, administered at 3 mg/kg by i.p., significantly decreased the duration of probe burying compared with saline, from 81.4 ± 19.7 s to 24.2 ± 10.8

s (P<0.005) (Fig. 4A), the number of head stretchings towards the probe (P<0.01) (data not shown), and the percentage of approaches toward the probe followed by retreats (P<0.01) (Fig. 4B).

Affinity of α_{s1}-casein tryptic hydrolysate and α-casozepine for $GABA_A$ receptor

Affinity for the BDZ site of $GABA_A$ receptor was tested because the tryptic hydrolysate produced the same effects as did drugs acting at the BDZ receptor (anticonvulsant activity, increase of the entries in the open arms without general activity change in the elevated plus-maze test, and modification of the conditioned defensive burying behavior). Anxiolytics that act through the 5-hydroxytryptamine receptors do not necessarily give positive results in the elevated plus-maze test (buspiron acts as an anxiogenic drug in this test (31). Displacement studies of [methyl-^{3}H]flunitrazepam showed that α_{s1}-casein tryptic hydrolysate interacted with the BDZ site of $GABA_A$ receptor with an IC_{50} of 72 μM and a Hill number of 0.8. The peptides of the α_{s1}-casein tryptic hydrolysate were prepurified by reversed-phase HPLC on C_{18} column in gradient conditions and lyophilized twice to remove TFA. The purified peptides were rechromatographied on a C_4 column in isocratic conditions, and the same lyophilisation procedure was carried out. All the peptides were recovered and tested in competition with [methyl-^{3}H]flunitrazepam. Only one fraction competed with flunitrazepam for the BDZ site of the $GABA_A$ receptor. This fraction contained only one peptide, of which the sequence was found to be Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg and the molecular mass, determined by FAB-MS, was 1266.6 Da. This corresponded to the α_{s1}-CN-(f91-100), consequently named α-casozepine, whose calculated molecular mass is 1266.1 Da. The two aromatic residues of this peptide allow determination of its concentration by spectrophotometry by using an ε value of 2810 cm^{-1}.liter.mol^{-1} at 275 nm. The calculated IC_{50} value for this peptidic fraction was 88 μM, and the Hill number was 0.8. The IC_{50} value for diazepam was 8.2 nM, and the Hill number was 1.2 under the same conditions. The peptide corresponding to α-casozepine was synthesized by using Boc solid-phase peptide-synthesis protocols, and the sequence was checked by Edman sequencing. The synthetic peptide, which displaced [methyl-^{3}H]flunitrazepam from the BDZ site of $GABA_A$ receptor with a calculated IC_{50} of 370 μM and a Hill number of 0.8, was about four times less potent than the natural one. The others peptides of the α_{s1}-casein tryptic hydrolysate had no affinity to the BDZ site of the $GABA_A$ receptor. None of them competed with the [methyl-^{3}H]flunitrazepam for this site. Consequently, the α_{s1}-CN-(f91-100) carries the entire anxiolytic activity of the hydrolysate.

α-Casozepine anxiolytic activity

α-Casozepine was tested in the conditioned defensive burying in order to confirm that it carries the anxiolytic activity of the α_{s1}-casein tryptic hydrolysate *in vivo*. The peptide administered i.p. at 0.4 mg/kg (0.32 μmol/kg) decreased significantly the duration of probe burying in comparison with saline (18.8 ± 13.7 s vs. 81.4 ± 19.7 s; P<0.005) (Fig. 4A). Other parameters such as number of head stretchings towards the probe (data not shown) and approaches towards the probe followed by retreats (Fig. 4B) were decreased too (P<0.005). The α_{s1}-CN-f(91-100) produced significant anxiolytic-like effects with efficiency similar to those of diazepam at 1 mg/kg (3.5 μmol/kg) i.p. in the conditioned defensive burying model.

α-Casozepine affinity for PBR

Affinity of α-casozepine for the MA-10 cell PBR was determined in competition with either [^{3}H]Ro5-4864 or [^{3}H]PK 11195. No displacement of ^{3}H-labeled ligands was observed, which indicated that α-casozepine did not bind to PBR and then was specific of the BDZ site of the $GABA_A$ receptor.

DISCUSSION

Cow's milk has long been considered a tranquilizing beverage with a sleep-inducing role, but the molecular bases of this belief are unknown. Cow's milk contains BDZ-like molecules (32), probably diazepam and its metabolites, but at very low concentration—between 0.5 and 2 μg/liter (33). As it seems unlikely that bovine tissues can synthesize heterocyclic ring with a chlorine atom, these substances would be either of external origin, such as mushroom grazing, or synthesized from plant precursors by rumen microorganisms. Such molecules are also found in human breast milk from women who do not take BDZs (34). The mean concentration of BDZ-like substances in human milk is 4.3 ± 2.3 μg/liter (35), whereas they are undetectable in women's blood. In the present work, we found evidence of a peptide resulting from bovine α_{s1}-casein hydrolysis with BDZ-like activity. α_{s1}-Casein was chosen because it is the major protein from bovine milk (36). Trypsin was used to hydrolyze α_{s1}-casein to approach an *in vivo* digestion, because it is one of the major proteolytic enzymes of the gastrointestinal tract. Anxiolytic molecules, such as BDZs, contain several aromatic rings. This finding potentially eliminated chymotrypsin (EC 3.4.21.1) and pepsin (EC 3.4.23.1), two other major physiological enzymes, of which preferential cleavage sites include aromatic residues. Activity of pepsin is very low in the newborn during the first 3 weeks (37) due to a stomach pH higher than 5, and trypsin is the only protease of which concentration is similar to that in adults (38).

In the present study, we have shown that i.p. injection of α_{s1}-casein tryptic hydrolysate significantly reduced PTZ-induced seizures in Wistar rat. PTZ greatly reduces chloride-dependent responses to the iontophoresis of transmitters (39). PTZ modifies GABAergic mediation because it leads to a total or partial inhibition of GABA-induced $^{36}Cl^-$ uptake in primary cultures of cortical neurons (40). PTZ also increases the concentration of glutamate and decreases that of GABA (41) because it is involved in the inhibition of glutamate dehydrogenase (EC 1.4.1.3) and aspartate transaminase (EC 2.6.1.1) and in the stimulation of GABA transaminase (EC 2.6.1.19). It has been shown recently that an acute injection of PTZ decreased $GABA_A$ receptor γ2, α1, and β2 subunit-mRNAs in cerebral cortex and cerebellum and affected the coupling mechanism between the GABA and BDZ sites of the $GABA_A$ receptor (42). PTZ administered chronically in rodents induces kindling, and these animals show an enhanced susceptibility to convulsions induced by different inhibitors of central GABAergic function. PTZ may be then associated with a persistent reduction in the inhibitory function of the GABAergic system in the brain (43), and PTZ-induced seizures can be considered as a model of epilepsy mediated through a specific interaction with the GABA-gated chloride ionophore. Diazepam and medazepam have a marked anticonvulsive effect on the clonic-tonic convulsions in PTZ-kindled rats (44). After i.p. injection of α_{s1}-casein tryptic hydrolysate, we have observed a reduction of the crisis severity and an increase of the crisis latency, which were similar to the effect of

diazepam (2 mg/kg) i.p. injection (data not shown). The hydrolysate would modulate the GABAergic transmission. Although the injected dose of PTZ to nonkindled animals was an acute convulsive dose (the mean stage 4 dose of PTZ in nonkindled rats is 75 mg/kg, 45), it caused seizures in only 63% of the rats. DMI used to dissolve the tested molecules had a slight protective effect against PTZ-induced seizures, so the injected dose of PTZ can be considered as a subconvulsant dose. The repeated injections of such a subconvulsant dose during the five experiments should have produced a progressive sensitization to the effects of PTZ in the animals, which became kindled (46). It has been shown that doses ineffective after the first injection induce tonic seizures after 20 injections (47) and that kindling effect is proportional to the dose of PTZ (48). The decrease of crisis latency and the increase of clonus duration among the control experiments show that kindling increased sensitivity to PTZ convulsant effects. Consequently, the protective effect of the α_{s1}-casein tryptic hydrolysate against PTZ-induced seizures was probably minimized.

The elevated plus-maze test was chosen to measure anxiolytic effect of the α_{s1}-casein tryptic hydrolysate. Brain serotonergic, noradrenergic, and GABAergic mechanisms are involved in the regulation of conflict behavior; but the $GABA_A$/BDZ receptor complex plays the most central role in this context. In the elevated plus-maze paradigm, diazepam reduces open-arm avoidance; whereas flumazenil, a BDZ antagonist, has no significant behavioral effect (49). In the present experiment, a reduced open-arm avoidance was observed with diazepam and α_{s1}-casein tryptic hydrolysate because the ratio of entries into the open arms to the total number of arm entries enhanced by comparison with control. This parameter is reliable in assessing the anxiolytic activity of drugs so the elevated plus-maze is commonly used. The standard measures sometimes give false–negative results, as with BDZs injected to animals habituated to gentle handling or tested in less-aversive conditions (50). On the contrary, no false–positive results have been reported, and this finding strongly supports the anxiolytic-like activity of the α_{s1}-casein tryptic hydrolysate. The conditioned defensive burying paradigm was then chosen to verify the anxiolytic effect of the hydrolysate. When rats are electrically shocked once through a stationary probe, they then bury it with material from the floor of experiment chamber. This “conditioned defensive burying” response is dose-dependent, reduced by various anxiolytic drugs (51). Full and partial BDZ agonists decrease the probe burying duration, which is the crucial parameter (52). BDZ antagonists, such as flumazenil, do not affect the duration of burying (53), whereas BDZ partial inverse agonists lead to a reduction of burying. So, other parameters are needed to differentiate agonists from partial inverse agonists. Full agonists, such as diazepam, increase exploratory approaches to the probe and decrease escape movements from the probe (decrease of the percentage of approaches toward the probe followed by retreats), whereas partial inverse agonists produce a decrease of exploratory approaches to the probe without changing the frequency of retreats (increase of the percentage of approaches towards the probe followed by retreats) (52). A decreased probe-burying duration and a decreased percentage of approaches towards the probe followed by retreats were observed in the α_{s1}-casein tryptic hydrolysate-treated group. The diazepam-like profile observed in the elevated plus-maze paradigm and in the conditioned defensive burying experiment revealed an anxiolytic-like activity of the α_{s1}-casein tryptic hydrolysate consistent with its binding to the BDZ site of the $GABA_A$ receptor. Only one peptide of the tryptic hydrolysate, the α_{s1}-CN-(f91-100), displaced [methyl-^{3}H]flunitrazepam from $GABA_A$ receptor. The synthetic peptide displayed the same activity, confirming that α-casozepine carries the total anxiolytic activity.

The fact that the synthetic peptide was less active *in vivo* and *in vitro* than the natural one has already been observed with biological active peptides prepared from caseins (54), but this finding is not explained. In the conditioned defensive burying paradigm, the α_{s1}-CN-(f91-100) displayed the anxiolytic activity observed with the α_{s1}-casein tryptic hydrolysate. An i.p. injection of 0.4 mg/kg of peptide in the rat led to the same behavioral results than an i.p. injection of 1 mg/kg of diazepam. Considering the difference in the molecular weight, the α_{s1}-CN-f(91-100) might be about 10 times more active than diazepam *in vivo*, whereas it was less affine than diazepam for the BDZ site of the $GABA_A$ receptor *in vitro*. Some BDZs, such as diazepam or flunitrazepam, exhibit relatively high affinity for the BDZ site of the $GABA_A$ receptor and for the PBR, which may play a part in anxiolytic response via the steroidogenesis regulation (55). However, the important *in vivo* anxiolytic activity of the α_{s1}-CN-(f91-100) cannot be explain by such a mechanism because this peptide exhibited no affinity for the PBR and did not significantly affect the progesterone production by MA-10 Leydig cells (data not shown). Changes in peptide conformation due to microenvironment modifications could also be also hypothesized to explain the difference between the *in vivo* and *in vitro* activity of α-casozepine.

Until now, investigations to identify an endogenous agonist ligand of the BDZ site of the $GABA_A$ receptor have failed (56). However, an 86-residue peptide called DBI, first isolated from rat brain (57), *in vitro* inhibits competitively the binding of BDZs to their receptor with a Ki of 4 μM. DBI is the precursor of two molecules named octadecaneuropeptide (ODN) (DBI-(f33-50)) and triakontatetraneuropeptide (TTN) (DBI-(f17-50)) carrying BDZ receptor binding activity. *In vivo*, DBI and its active fragments are anxiogenic (58). DBI also displays epileptogenic activity and seems to be a peripheral marker of epilepsy (59). The comparison of bovine DBI and α_{s1}-CN-(f91-100) sequences by the CLUSTALW procedure (Fig. 5) leads to an alignment in the carboxy-terminal region of the DBI (residues 73 to 82). Three residues are identical and four residues are homologous. According to Andersen et al. (60), DBI-(f66-83) corresponds to a helical region with potential amphipathic properties. Whereas only 15% of the α_{s1}-casein residues are in α-helixes, α_{s1}-CN-(f91-100) belongs to a helical region (61). Curiously DBI-(f73-82) and α_{s1}-CN-(f91-100) have a similar primary structure and share the same secondary structure. This DBI region does not belong to the ODN or the TTN that bind on BDZ site of the $GABA_A$ receptor. The DBI-(f73-82) injected at 1 mg/kg i.p. in the Wistar rat displayed a significant anxiolytic activity in the plus-maze experiment (data not shown).

In conclusion, an anticonvulsant and anxiolytic molecule not structurally related to BDZ could be formed from bovine casein during digestion. The α_{s1}-CN-(f91-100) peptide bound to the BDZ site of the $GABA_A$ receptor but not to PBR. The physicochemical characteristics and the pharmacological action of α-casozepine lead us to hypothesize that this peptide might play a role, as an external ligand, in the regulation of the nervous system of the mammalian newborn and might be involved in the traditional calming properties attributed to milk.

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Fig. 1

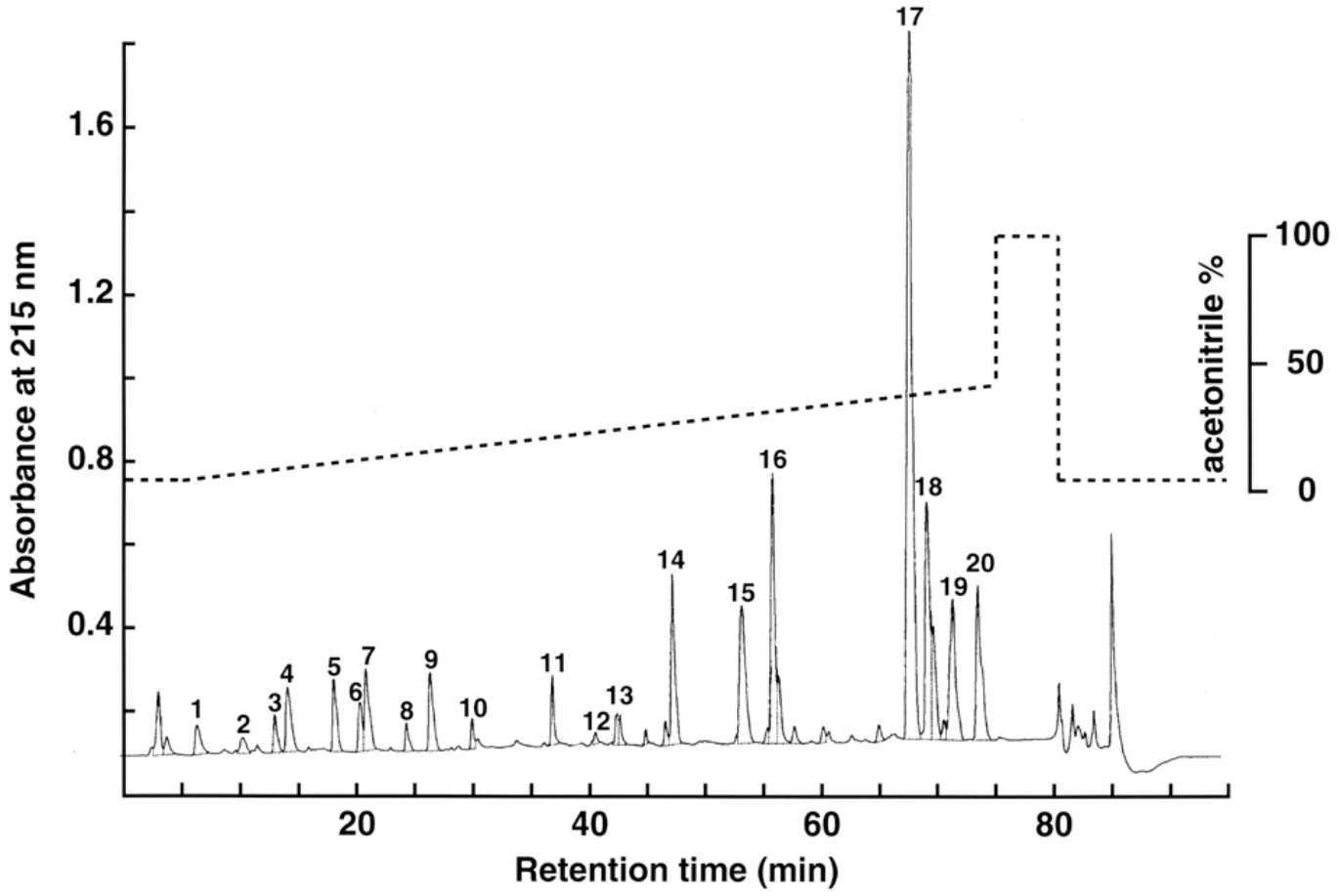

Figure 1. Separation of α_{s1}-casein tryptic hydrolysate peptides by reversed-phase HPLC on a C18 column at room temperature. The gradient (dotted line) was 5%–40% of acetonitrile in water containing 0.1% (v/v) TFA for 70 min at a flow-rate of 1 ml/min. Injection was of 400 µg of hydrolysate. Peptide identification: peak 1=α_{s1}-CN-(f1-3), peak 2=α_{s1}-CN-(f101-103), peak 3=α_{s1}-CN-(f80-83), peak 4=α_{s1}-CN-(f4-7), peak 5=α_{s1}-CN-(f125-132), peak 6=α_{s1}-CN-(f84-90), peak 7=α_{s1}-CN-(f120-124), peak 8=α_{s1}-CN-(f35-42), peak 9=α_{s1}-CN-(f80-90), peak 10=α_{s1}-CN-(f59-79), peak 11=α_{s1}-CN-(f43-58), peak 12=α_{s1}-CN-(f35-58), peak 13=α_{s1}-CN-(f106-119), peak 14=α_{s1}-CN-(f104-119), peak 15=α_{s1}-CN-(f8-22), peak 16=α_{s1}-CN-(f194-199), peak 17=α_{s1}-CN-(f91-100) and α_{s1}-CN-(f152-193), peak 18=α_{s1}-CN-(f23-34), peak 19=α_{s1}-CN-(f125-151), peak 20=α_{s1}-CN-(f133-151).

Fig. 2

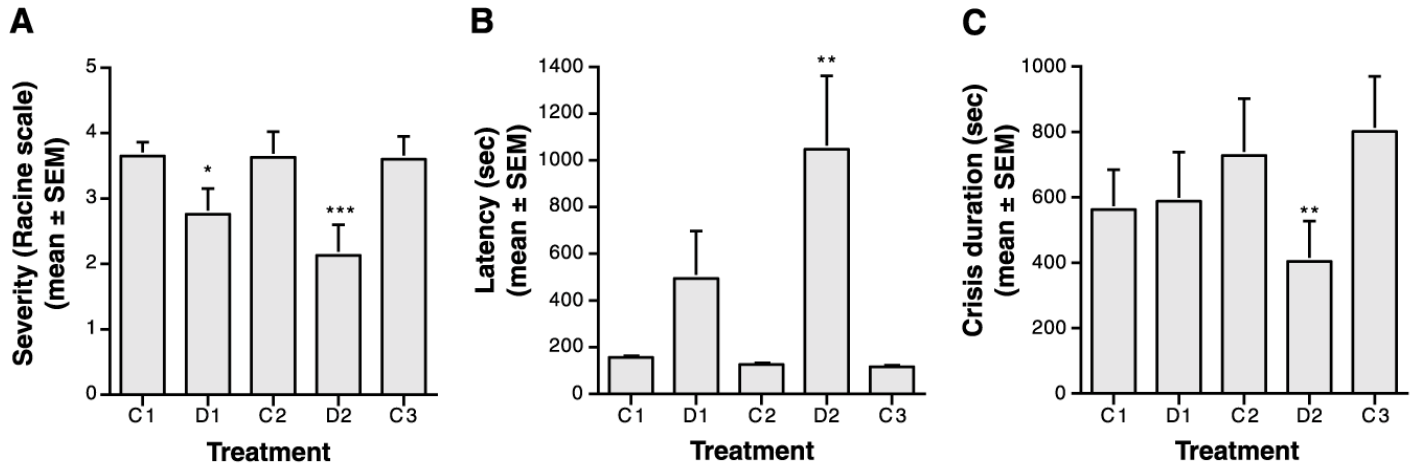

Figure 2. Effect of the α_{s1}-casein tryptic hydrolysate on the severity (A), latency (B), and duration (C) parameters of a crisis induced by i.p. injection of 60 mg/kg of pentylenetetrazole dissolved in 9‰ NaCl in Wistar rats. The same animals (n = 17) were used for all the experiments. 2 ml/kg of a solution of 25% (v/v) DMI in water (controls C1, C2, and C3), 1 mg/kg (D1), or 3 mg/kg (D2) of α_{s1}-casein tryptic hydrolysate were injected by the i.p. route 30 min before the pentylenetetrazole injection. For the severity parameter **(A)**, values are the mean of crisis intensity according to Racine SE. For the latency parameter **(B)**, values are the mean of the time (seconds) needed to observe the first parameter of crisis ± SE (if no crisis parameter was observed a value of 2700 s was taken in account). For the duration parameter, values are the mean of the total time (seconds) of crisis±SE. * $P < 0.02$; ** $P < 0.005$; *** $P < 0.002$ by repeated measures ANOVA procedure corrected by Greenhouse–Geisser epsilon.

Fig. 3

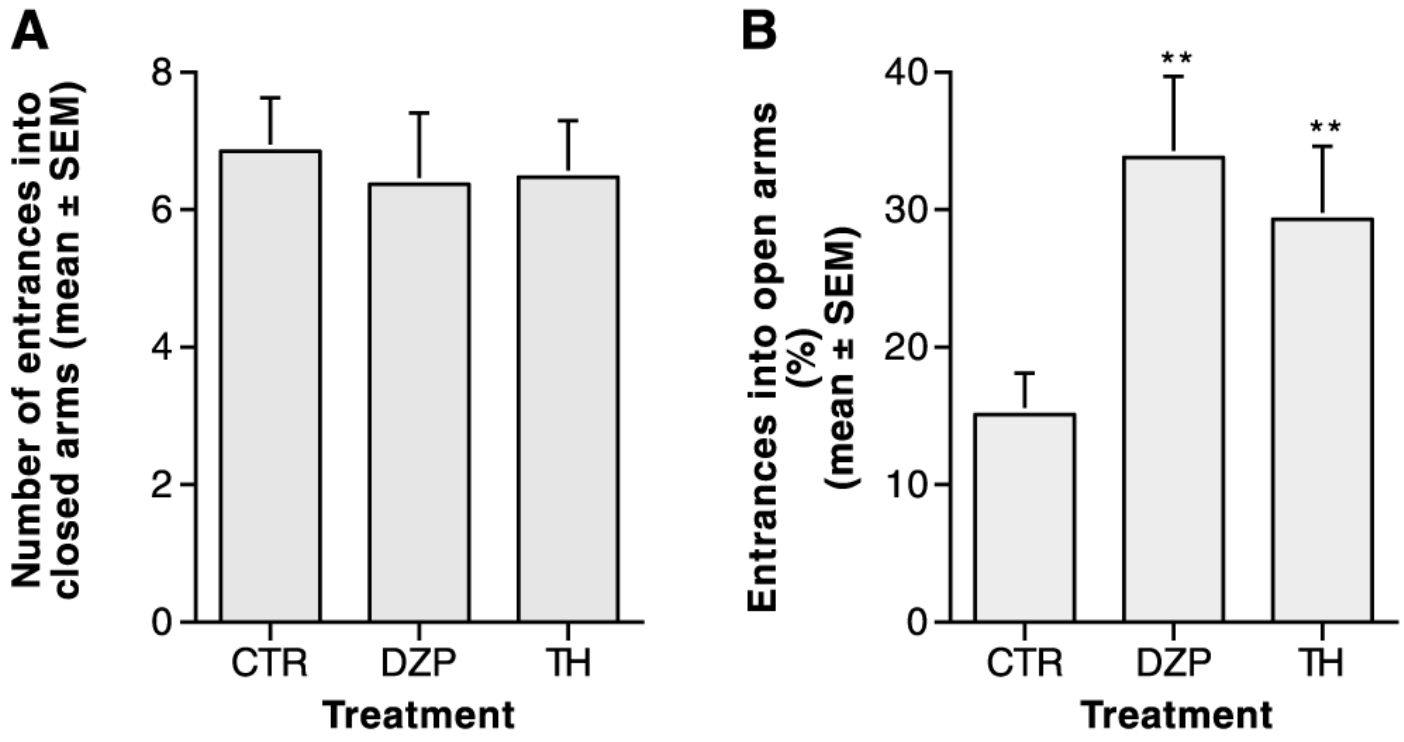

Figure 3. Effect of the α_{s1}-casein tryptic hydrolysate on the performance of Wistar rats in the elevated plus-maze paradigm compare to diazepam. 9‰ NaCl (CTR), 1 mg/kg of diazepam (DZP) or 3 mg/kg of α_{s1}-casein tryptic hydrolysate (TH) were injected by i.p. route 30 min. before experiment. Behavior of animals (n=20, each group) was tape-recorded during 5 min. Values are **(A)** the mean±SE of the number of entrances into the closed arms of the plus-maze, **(B)** the mean±SE of the percentage of entrances into the open arms (entrances into the open arms to the total entrances). ** $P < 0.02$ by ANOVA procedure.

Fig. 4

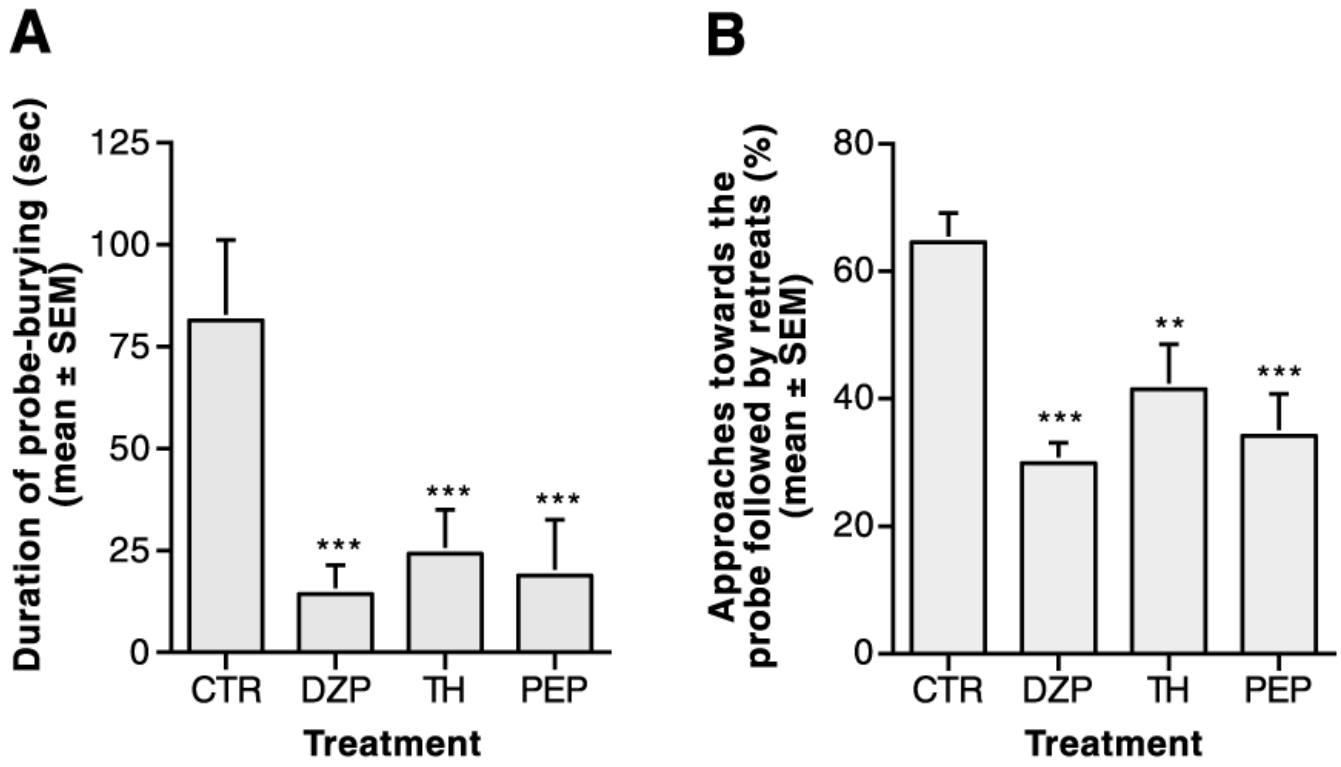

Figure 4. Effect of the α_{s1}-CN-(f91-100) and α_{s1}-casein tryptic hydrolysate on conditioned defensive burying in the Wistar rat by comparison to diazepam. 9‰ NaCl (CTR), 1 mg/kg of diazepam (DZP), 3 mg/kg of α_{s1}-casein tryptic hydrolysate (TH) or 0.4 mg/kg of α_{s1}-CN-(f91-100) (PEP) were administered by i.p. way (*n*=12, each group), 30 min before the burying test commenced. Behavior of animals was tape-recorded during 5 min. Values are **(A)** the mean ± SE of time (s) spent to bury the probe, **(B)** the mean ± SE of the percentage of approaches towards the probe followed by retreats (number of retreats to number of approaches). ** $P < 0.01$; *** $P < 0.005$ by ANOVA procedure.

Fig. 5

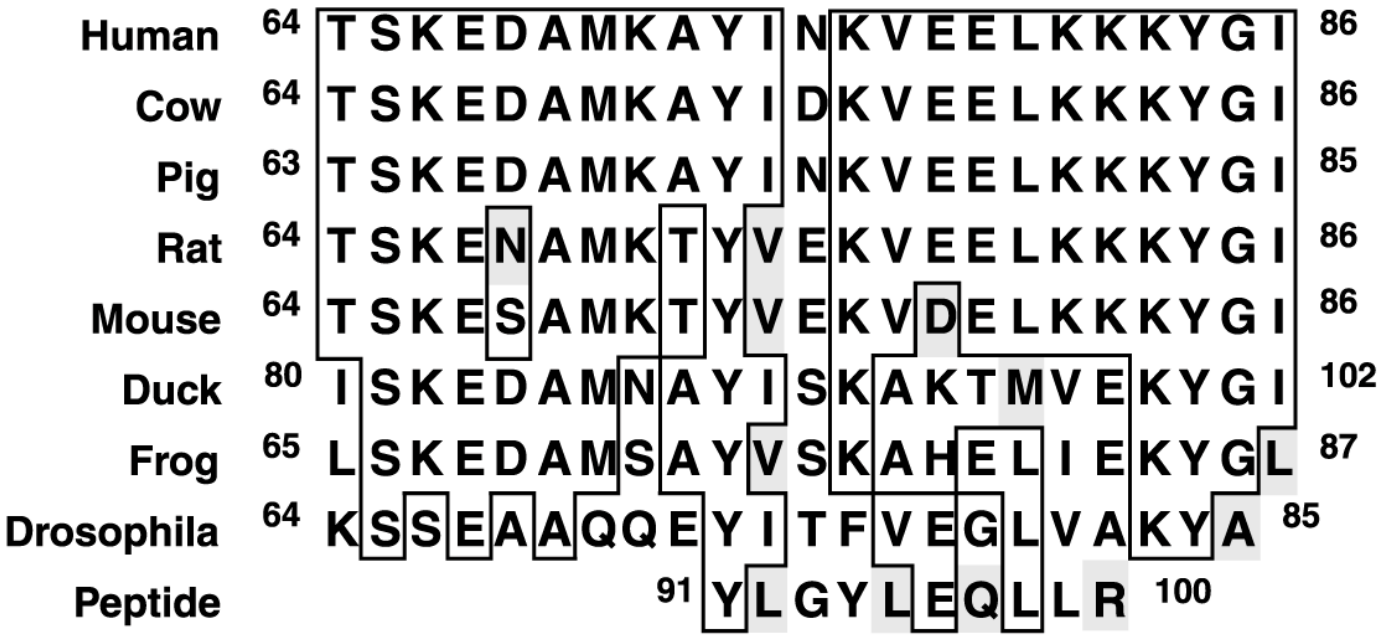

Figure 5. Alignment of α_{s1}-CN-(f91-100) with sequences of DBI carboxy-terminal part from human (*Homo sapiens*) (P07108), cow (*Bos taurus*) (P07107), pig (*Sus scrofa*) (P12026), rat (*Rattus norvegicus*) (P11030), mouse (*Mus musculus*) (P31786), duck (*Anas platyrhyncos*) (P45882), frog (*Rana ridibunda*) (P45883) and drosophila (*Drosophila melanogaster*) (P42281). Alignment was performed with CLUSTALW multiple alignment method. Identities are boxed; similarities are shaded without boxing.

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Modulation of Cerebral Activity Induced by α-casozepine, a Benzodiazepine-like Peptide Derived from Bovine Casein

Simon Benoit, Catherine Chaumontet, Céline Cakir-Kiefer, Daniel Tomé, and Laurent Miclo

Published Online: 1 Apr 2016 **Abstract Number:** 125.4

Abstract

The tryptic hydrolysate of bovine α_{s1}-casein (CH) displays anxiolytic properties highlighted in several animal species and in humans. Unlike benzodiazepines (BZD), the most prescribed anxiolytic drugs, CH shows neither addiction, dependence, sedation nor toxicity. The search for a carrier bioactive molecule within CH led to the α-casozepine (α-CZP), a decapeptide which anxiolytic properties were confirmed in rats. Its affinity for the benzodiazepine site of the $GABA_A$ receptor has helped getting the α-CZP closer to the BZD family, despite a much lower affinity than the BZD reference diazepam. The aim of this study was to characterise the changes in the activity of the brain areas involved in the reduction of anxiety after intraperitoneal administration of α-CZP by labelling neuronal activity in mice brain, in order to characterise its mechanism of action.

Swiss mice were fed with a soy-protein based diet containing no caseins, in order to prevent the presence or potential release of bioactive peptides from these milk proteins. Animals (8 per group) were placed in an anxiety-producing situation (light-dark box) 30 minutes after an intraperitoneal injection of α-CZP (1 mg/kg), diazepam (1 mg/kg) or of the vehicle used to solubilize the molecules. The molecules were then perfused with formalin 1h30 after this stimulus. Brain expression of c-Fos (a marker of neuronal activity) was measured by automatic counting with immunofluorescence on sagittal and coronal brains sections.

The anxiolytic effects of α-CZP on mice were confirmed using the light-dark box (significant augmentation of the time spent in the aversive lit box). Immunofluorescence analysis showed a significant lower expression of c-Fos in the prefrontal cortex (−60%), hippocampus (−40%), nucleus accumbens (−50%) and hypothalamus (−60%) after administration of the α-CZP compared to the vehicle. The same profiles were observed after diazepam injection. A significant increase in the expression of c-Fos in the amygdala (+300%), observed only after the administration of α-CZP, indicates a different mechanism of action compared to diazepam. Results were confirmed on coronal sections.

In conclusion, this study showed that an intraperitoneal administration of α-CZP, a bioactive peptide resulting of a food protein hydrolysis, allows a modulation of neuronal activity in different brain regions involved in the regulation of anxiety and thereby can partly explain the anxiolytic activity of the peptide. A binding of α-CZP on BZD receptors could explain the diminution of neuronal activity in different brain regions associated with the anxiolytic effects of the peptide. Moreover, a different mechanism of action of

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Short-Term Anxiolytic and Pro-Hypnotic Activity of a Tryptic Hydrolysate of Bovine As1-Casein in Patients with Anxiety Spectrum Disorders

Giuseppe Ceraudo[1]*, Sonia Cortopassi[1], Lavinia Rossi[1], Bernardo Dell'Osso[2,3] and Liliana Dell'Osso[1]

1 Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
2 Department of Psychiatry, University of Milan; Fondazione IRCCS Ca' Grande, Ospedale Maggiore Policlinico, Milan, Italy
3 Department of Psychiatry and Behavioral Sciences, Bipolar Disorders Clinic, Stanford University, CA, USA

Corresponding author: Giuseppe Ceraudo

beppeceraudo@yahoo.it

Clinica Psichiatrica, University of PisaVia Roma, 57 Pisa, Italy.

Tel: +39 3396739062

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Abstract

We conducted a prospective open-label study with 100 outpatients who had sought psychiatric consult in private clinical practice for anxiety/sleep in subthreshold/full blown DSM-IV Anxiety Spectrum Disorders. Clinicians, prescribed for 4 weeks a dietary supplement based on a formulation containing α-casozepine peptide 300 mg/day. The comparison of all rating scales mean scores reported at T0 versus T1 showed a statistically significant decrease ($p<0.001$). In Clinical Global Impression scale, the 54% of the sample was found to be much improved, 27% minimally improved and 19% showed no change. The 64% of the sample reported an anxiolytic effect, and among the 64 patients with sleep disorders, the 51.5% reported a pro-hypnotic effect. Considering patients in monotherapy with the dietary supplement, an anxiolytic effect was observed in 69.7% while a pro-hypnotic effect was observed in the 62.5% of the sample. No side-effects were reported during the treatment with no drop-out.

Keywords: α-casozepine; Dietary supplement; No side effects; Novel treatment

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Introduction

Anxiety Spectrum Disorders are amongst the most prevalent mental disorders and are responsible for reduced quality of life and significant disability in affected patients [1]. Effective treatment strategies for these conditions include psychotropic compounds (benzodiazepines and pro-serotonergic antidepressants) and psychotherapeutic interventions [2,3]. However, many patients do not have access to or refuse this kind of treatments for different reasons, including pharmacophobia, fear of dependence, economic and other motivations [4-6]. Therefore, novel nutraceutical compounds with potential anxiolytic effects have attracted the interest of researchers over the last years [7,8].

For generations, mothers have given their children a warm glass of milk before going to bed as a way to help them fall asleep. As far back as 1934, this home remedy gained scientific validation when it was observed that people who had taken milk and cornflakes were more likely to enjoy a full night of uninterrupted sleep [9]. Subsequently, Brezinova and Oswald showed, using electroencephalography, that sleep was significantly improved (longer and un-interrupted night sleep-time) in older people, when they had taken a combination of cow milk and cereals before going to bed [10]. In 1997, amongst pediatric researchers, Blass provided further evidence in the field by demonstrating that newborns given an infant formula containing milk fell asleep not solely due to nursing and being held, but specifically to something in milk itself [11]. Just at the beginning of the new millennium, it has been identified what that "something" was. It turned out that nutrients found in cow milk called bioactive peptides, chains of amino acids, exert a sedative effect on the brain and induce sustained sleep patterns [12]. These bioactive milk peptides have been shown to act on the brain GABA-A receptors, which represent the target of one of the most effective classes of sedatives: the benzodiazepines. Specifically, only one peptide named α-casozepine, corresponding to the 91–100 fragment from bovine αs1-casein, expressed affinity for GABA

A receptor [13]. In pre-clinical models, milk peptides markedly reduced anxiety and improved sleep in animals subjected to chronic stress [14]. Authors demonstrated that the injection of 3 mg/kg of α-casozepine significantly reduced the epileptic symptoms caused by pentylenetetrazole in rats [15]. An anxiety reduction was also observed when the hydrolysate was tested in the elevated plus-maze and in the conditioned defensive burying rat models. Using selective antagonists of 5-HT1A, D1 and GABA-A receptors, other authors [16] have demonstrated to inhibit α-casozepine anxiolytic effect, suggesting a synergic role of these receptors in the anxiolytic activity observed in mice. The α-casozepine amino acid sequence could be related to the carboxyterminal sequence of the polypeptide diazepam binding inhibitor, an endogenous ligand of the central GABA-A and peripheral-type benzodiazepine receptor, but α-casozepine activity was observed only in central GABA-A receptors [13]. More recently, in a double-blind placebo-controlled trial, α-casozepine showed improvements of stress-related symptoms in a total of 63 female volunteers suffering from at least one potentially stress-related disorder, such as anxiety, sleep problems and general fatigue [17]. After one month, the α-casozepine group significantly reduced their symptoms more than placebo, particularly in digestion, intellectual, cardiovascular, emotional and social problems. As widely known, also sleep disturbances benefit from psychotropic compounds with GABAergic activity. In this perspective, a representative sample of day-time workers from the general population of Japan, reporting insomnia during the preceding six months, was treated with α-casozepine in an observational study [18]. Results showed that α-casozepine improved sleep quality after two weeks of treatment and decreased the sleep latency and the daytime dysfunction after four weeks of treatment.

With the aim to investigate the anxiolytic and pro-hypnotic effect of α-casozepine, we conducted the present open-label prospective short-term study with 100 outpatients affected by Anxiety Spectrum Disorders and treated with α-casozepine for 4 weeks.

Material and Methods

We enrolled 100 outpatients who had sought psychiatric consult in private clinical practice for anxiety symptoms. All patients were screened using the Structured Clinical Interview (SCID-I) [19] to assess the psychiatric diagnosis at recruitment time, considering spectrum condition as a clinical picture which does not necessarily meet all the criteria requested for the diagnosis according to the DSM-IV-TR (APA, 2000) [20]. However, in terms of psychometric assessment, inclusion criteria required a minimum score of 8 at the Hamilton Anxiety Scale [21] and/or of 7 at the Insomnia Severity Index [22]. We considered only patients affected by Anxiety and Mood Spectrum Disorders with subthreshold or full-blown diagnoses in relation to reported anxiety symptoms. Three clinicians, according to patients' needs, proposed and prescribed a Dietary Supplement (DS) based on a formulation containing α-casozepine peptide at a dosage of 300mg/die (provided by Junia Pharma SRL, Italy). A written informed consent for participation into the study was provided by all patients, and the study protocol was approved by the Ethics Committee of the University of Pisa. With the aim to provide an evaluation over the course of anxious symptoms, we administered to all patients at recruitment time (T0) and after 4 weeks (T1) of treatment with DS the following rating scales: 1) the Hamilton Anxiety Scale (HAM-A), quantifying the severity of anxiety symptoms through the investigation of 14 specific items of the anxiety spectrum; 2) the Insomnia Severity Index (ISI), a rating scale for sleep disorders divided into seven multiple-choice questions on sleep quality and its influences in daily life; 3) the Clinical Global Impression scale (CGI) [23] indicating the overall clinical evaluation by a clinician in terms of severity and improvement, respectively, of the clinical picture as a whole. Finally, 4) the therapeutic evaluation was assessed using the Dosage Record and Treatment Emergent Symptom Scale (DOTES) [24], recording concomitant treatments and the presence of side effects due to the ongoing treatment.

Statistical analysis was performed using SPSS version 21 (USA). Qualitative and quantitative analysis of response were conducted. Considering a clinical response to treatment of anxious symptoms a decrease by half of the HAM-A and/or ISI score at T1 and patients who reported marked improvement to CGI. Response rate was compared according to the diagnosis and in patients who took DS in monotherapy versus add on treatment. Parametric variables were described as mean and standard deviation (SD) and were compared by paired t-test or independent t-test. Categorical variables were compared by $\chi 2$ test. Statistical significance was assigned for $p<0.05$.

Results

Of the 100 outpatients reporting anxiety symptoms (70 females, mean age 39 + 14.7), 53% met a diagnosis of subthreshold Anxious Spectrum Disorder (patients with anxious symptoms not meeting all the criteria necessary for diagnosis of Anxiety and/or Mood Disorders, according to DSM-IV-TR), 20% had Sleep Disorders, 16% Panic Disorder and 11% were affected by Bipolar Disorders **(Table 1).**

After 4 weeks of treatment, it emerged that 54% of our sample showed a marked improvement at the CGI scale and 27% was found to be minimally improved, while 19% had no change. The comparison of CGI score (T1 versus T0) documented a statistically

Table 1 Demographic and clinical features of the study samples.

Variables	Overall Sample (n=100)	%
Mean age	39 years (18-78) ds=1.47	
Gender	Females	70%
Occupation	Employed	60%
	Student	20%
	Unemployed	7%
	Retired	13%
Diagnosis	Anxiety Spectrum Disorders	53%
	Sleep Disorder	20%
	Panic Disorder	16%
	Bipolar Disorder	11%
Treatment	Lactium monotherapy	43%
	Lactium as add-on treatment	57%

Table 2 Rating scales mean score course at T_0 and T_1.

HAM-A	12.96 ds=6.79	7.21 ds=6.19	13.8	<0.0001
ISI	8.45 ds=6.48	4.91 ds=5.19	9.44	<0.0001
CGI	3.31 ds=0.80	2.54 ds=0.95	10.86	<0.0001

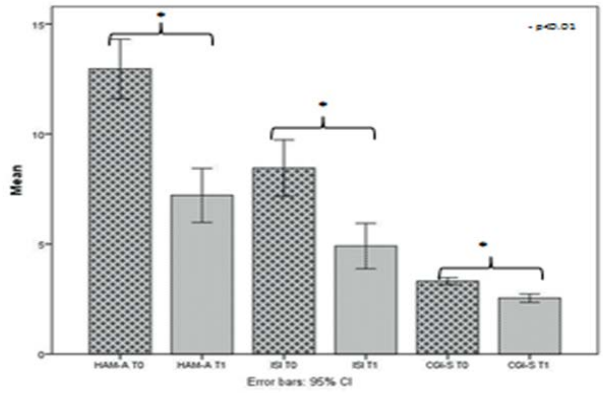

Figure 1 Rating scales mean score course at T_0 and T_1.

significant reduction of the mean scores (3.31 ± 0.80 vs 2.54 ± 0.95, p<0.0001). Moreover, we observed a significant reduction of the HAM-A mean scores comparing T1 vs T0 (12.96 ± 6.79 vs 7.21 ± 6.19, p<0.0001). Of the 64 patients with insomnia, the ISI mean score showed a significant decrease from T0 to T1 (8.45 ± 6.48 vs. 4.9h ± 5.19, p<0.0001) **(Table 2 and Figure 1).**

Considering a clinical response to treatment of anxious symptoms a decrease by half of the HAM-A and/or ISI score at T1, we documented a clinical response in the 64% of our sample. When comparing patients taking DS monotherapy (n=43) versus add-on treatment (n=57) (predominantly SSRI or SNRI antidepressants and mood stabilizers), we observed a clinical response in 69.7% of those in monotherapy (HAM-A score) at T1 versus 59.6% who took DS in add-on to other treatments without statistically significant differences.

With regards to the 64 patients with sleep disorders, the 51.5% had a response in terms of ISI score at T1. Patient with sleep disorders and DS in monotherapy represented the 24% of the sample and 62.5% of them showed a clinical response in terms of ISI reduction at T1 while only the 45% of patients who took DS with other treatments, showed a clinical response (p>0.05).

According to the diagnosis, the presence of Panic Disorder was found to be associated with a higher (though not statistically significant) response to treatment than Bipolar Disorder and Anxious Spectrum Disorders respectively (75% vs 72% vs 53%). The analysis of the Dotes scores revealed that DS was not associated with any side effects.

Discussion

In our sample of 100 patients with Anxiety Spectrum Disorders treated with a dietary supplement based on a formulation containing α-casozepine peptide 300 mg/day for 4 weeks, 64% reported an anxiolytic effect, and of the 64 patients with sleep disorders 51,5% reported a pro-hypnotic effect. Considering patients in monotherapy with DS, an anxiolytic effect was observed in 69,7% and 62,5% showed pro-hypnotic effect. The comparison of all rating scales mean scores, at T1 versus T0, showed a statistically significant decrease. Of note, no side-effect was detected in relation to DS treatment. From the analysis of these open-label data, it emerged that DS may be helpful to treat mild/moderate anxiety symptoms. Present findings seem consistent with the results of a recent study reporting sedative and anxiolytic-like effects in pentobarbital-induced sleeping behavior of mice, after the administration of milk collected at night [25].

Serotoninergic antidepressants and benzodiazepines are the most prescribed pharmacological treatment in anxiety disorders [26-28]. Even though in appearance they have two distinct mechanisms of action, serotoninergic antidepressants inhibit glutamatergic neurons of the amygdala through increased extracellular serotonin levels [29]. This inhibition engenders anti-anxiety action. Benzodiazepines also inhibit the amygdala facilitating GABAergic activity, thereby reducing fear and anxiety. The inhibitory action on the amygdala might be, therefore, a common mechanism of anti-anxiety treatments [29]. However, both treatments induce different side effects, serotonergic antidepressants being often responsible for sexual dysfunctions, nausea and weight gain [30], whereas benzodiazepines for tolerance/dependence, somnolence and cognitive deficits [31]. With the aim to assess the evidence about the efficacy of antidepressants and benzodiazepines in adults with Panic Disorder, a recent review analyzed 35 double-blind randomized controlled trials, including 6785 participants overall [32]. Authors found low-quality evidence suggesting no difference between antidepressants and benzodiazepines in terms of response rate. In addition, very low-quality evidence suggested a benefit for benzodiazepines compared to antidepressants in terms of dropouts due to any cause, even if confidence interval ranges from almost no difference to benefit with benzodiazepines. From this perspective, it clearly emerges the need to develop additional treatments with proven efficacy and tolerability in anxiety disorders like Panic Disorder. Even psychotherapeutic interventions are not always easily accessible for patients with anxiety disorders for economic motivations or in light of lack of trust in the therapist or to the effectiveness of treatment [33].

Nutraceutical compounds represent a novel area in a new era of therapeutics for Anxiety and Mood Disorders, in which natural compounds, generally safe and well tolerated, may become a valid treatment option in monotherapy or in add-on to benzodiazepines/antidepressants [34]. Patients frightened by psychopharmacological treatments, patients reporting side-effects beyond minimal dosages, subjects with poor adherence or with unsatisfactory response to treatment can benefit from natural compounds. In particular, those which contains α-casozepine might be used in the acute treatment phase of Anxiety Disorders in add-on to antidepressants and/

or benzodiazepines as well as in monotherapy in prodromal or in maintenance phases and to treat residual symptoms. The potential advantages, which need to be assessed under controlled conditions, may be represented by a greater containment of side effects for lower dosages and therefore a greater adherence to ongoing treatment, from a reduction of the duration of overall treatment and a greater ease to their suspension. In addition, the possibility of being able to prescribe DS to special populations like elderly, child/adolescents, pregnant women and patient with conditions of poor health might be worthy of specific evaluation.

Furthermore, from this study it emerged a double effect, anxiolytic and pro-hypnotic, attributed to DS that misses to antidepressants and that benzodiazepines tend to lose overtime. A dietary supplement based on a formulation containing α-casozepine could be a valid option in the treatment of anxiety spectrum disorders in monotherapy or in add-on to serotoninergic antidepressants and/or benzodiazepines.

In the interpretation of the presented results, the following methodological limitations need to be taken into account. The study was not conceived as a controlled randomized study and it was specifically focused on the short-term treatment. In addition, the studied population was not homogenous in terms of diagnosis, severity, comorbidities and concomitant treatment, being composed by diagnostic subgroups that in some cases were too small in order to detect a statistically significant difference. Nonetheless, we believe that the present uncontrolled report may pave the way for future controlled investigation of the anxiolytic and pro-hypnotic effects of DS in patients with Anxiety Disorders.

Conclusion

The present open-label study found a significant short-term anxiolytic and pro-hypnotic effect of DS in patients with Anxiety Spectrum Disorders, and in subjects with Panic Disorder and Sleep Disorders. The absence of side effects of this natural compound can improve the compliance to treatment, and might be worthy of investigation amongst special populations like elderly, child-adolescents and pregnant women. Future double-blind, randomized controlled studies are needed to evaluate the efficacy and safety of DS treatment in the long-term and in different populations.

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Conflicts of Interest

The authors report no conflicts of interest in relation to the content of the present study.

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↓ Full text

Therapeutic effects of an alpha-casozepine and L-tryptophan supplemented diet on fear and anxiety in the cat.

Landsberg G, et al. J Feline Med Surg. 2017.
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Abstract

Objectives This study assessed the anxiolytic effectiveness of a test diet (Royal Canin Feline Calm diet) supplemented with L-tryptophan and alpha-casozepine. Methods Subjects were 24 cats that were classified as mildly or markedly fearful based on the presence of a person in their home room. Three different protocols were used to assess anxiety: (1) evaluation of the response to a human in the cat's home room (home room test); (2) analysis of the response to placement in an empty test room (open-field test); and (3) analysis of the response to an unfamiliar human (human interaction test). All three protocols were first run at baseline, and the results were used to assign the animals to control and test diet groups that showed equivalent fear and anxiety. Both groups were retested on the three protocols after 2 weeks (test 1) and again after 4 weeks (test 2). Results The diet groups differed for two behavioral measures in the open-field test: inactivity duration and inactivity frequency. The control group showed statistically significant increases in inactivity duration between baseline and test 1 and baseline and test 2, while the group fed the test diet showed a marginally not significant decrease in inactivity duration between baseline and test 1 and a not significant decrease for test 2. There was also a significant increase in inactivity frequency between baseline and test 1 in the test diet group and marginally not significant decrease in the control group. There were no differences between groups in the approach of the cats toward people for the home room test and the human interaction test. Conclusions and relevance These results suggest that the test diet reduced the anxiety response to placement in an unfamiliar location, but that fear in the presence of an unfamiliar person was not counteracted by the diet.

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