Food protein-derived anxiolytic peptides: their potential role in anxiety management

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Abstract

About one in three people are affected by anxiety disorders during their lifetime. Anxiety episodes can be brief due to a stressful event, but anxiety disorders can last at least 6 months. A wide variety of therapeutic drugs are available for the treatment of anxiety disorders, but due to the associated side effects of these anxiolytics, it is interesting to find alternatives. Some food protein hydrolysates or active peptide fragments present in such hydrolysates provide a natural and promising mean for preventing certain forms of anxiety. To date, only a small number of hydrolysates or peptides from food proteins with anxiolytic-like activity have been characterized. Most of these hydrolysates or peptides have displayed potent anxiolytic profiles in animal or clinical studies. The results suggest that these molecules may exert their effects at different levels. This paper reviews the data of the structure/activity relationship, physiological effects displayed in in vitro and in vivo assays, bioavailability, and safety profiles of anxiolytic peptides.

1. Introduction

Anxiety disorders are the most prevalent mental disorders and constitute a substantial burden globally due to associated immense health care expenditures. They are characterized by feelings of anxiety and fear (worries about the future and present, respectively) with physical symptoms interfering with normal life activities.[1] A European epidemiological study of mental disorders indicates that one third of the population is affected by an anxiety disorder during their lifetime.[2] The prevalence rates of 12-month (3.3–20.4% of population) and lifetime (5–25%) anxiety disorders vary across the countries.[3] According to the latest estimates of the Institute for Health Metrics and Evaluation, about 5.4% (25 million people) of the European population is living with anxiety disorders and they are most common in women than men.[4] These disorders have been shown to have high serious consequences on work efficiency and often lead to job loss.[5] Other comorbidities or psychiatric disorders such as alcohol abuse, drug addiction and depression (representing 2.3%, 1.8% and 11.0%, respectively, of study population) are highly associated with anxiety disorders as their prevalence multiplied by 2 or 3 if they co-occur with anxiety disorders.[6,7] Many anxiolytics (ethanol, barbiturates, thalidomide, meprobamate, benzodiazepines (BDZs), buspirone, *etc.*) were introduced along time to treat anxiety disorders. Nevertheless, some of the drugs were quickly withdrawn from the market due to their teratogenic effects (thalidomide) while many others caused strong side effects (*e.g.* ethanol or barbiturates). BDZs remain the primary treatment of choice and are generally prescribed to treat or mitigate anxiety disorders. Nonetheless, the BDZ treatment duration is usually longer (5 months) than recommended, *i.e.* 12 weeks.[8] Moreover, their long-term usage involves adverse effects (tiredness, nausea, cognitive impairment, paradoxical excitement, hostility, confusion, behavioral disinhibition and depression) and provides evidence for tolerance development to their anxiolytic, anticonvulsant and sedative actions.[9] Therefore, there is growing scientific and commercial interest to find out novel anxiolytic agents with better safety profiles than BDZs. In this context, the investigation of natural sources has become a centre of interest.

Recent scientific studies have reported that food proteins not only serve as nutrients, but also perform various health promoting physiological roles, which are primarily due to peptide sequences encrypted in the parent protein. They are inactive within the structure of the native protein, but once released from this protein by proteolytic enzymes *in vitro* or during processing by food exogenous or endogenous proteases or by digestive proteases during gastrointestinal passage, they could exert their beneficial effects in the consumer's body.[10–12] Therefore, bioactive peptides have been defined as specific protein fragments that have a positive impact on the body functions and may ultimately influence health.[13] Currently, more than 1500 bioactive peptides have been reported in the "Biopep" database.[14] The size of active sequences commonly varies from 2 to more than 20 amino acid residues – up to 64 residues in the caseinomacropeptide from bovine κ-casein – and some peptides are known to demonstrate multi-functional properties.[10,15]

To date, many dietary proteins from animal and plant sources have been widely studied as the source of bioactive peptides, which play a significant role in human health by affecting the key body systems such as cardiovascular, digestive, endocrine, immune, skeletal, and nervous systems.[10–12] Their bioactivities such as antihypertensive, antioxidant, antimicrobial, immunomodulatory, antidiabetic and mineral binding activities have been described in many articles.[16–20] However, there are few peptides from a limited number of dietary sources (such as milk, soybean, fish, spinach and egg) that exhibit an anxiolytic activity (Fig. 1). This activity is original as it has been demonstrated in *in vivo* models and has never been reviewed. The purpose of using biologically active peptides with anxiolytic activity is not to replace anxiolytic drugs, but these peptides may in some cases allow managing anxiety situations and, moreover, they could lead to the design of new families of anxiolytic molecules. Therefore, in this review we discuss these peptides.

2. Milk protein-derived anxiolytic peptides

Milk is a rich source of proteins comprising caseins and whey proteins. Caseins account for 80% of the total bovine milk protein inventory and 20–45% of human milk.[20] In the last three decades, it has been shown that milk proteins are a source of bioactive ingredients that impart extra-nutritional benefits. Some of the health benefits (*e.g.* inhibition of thrombin-induced platelet aggregation and serotonin secretion *in vitro*) have been evidenced for native caseins while others appear only after their proteolysis leading to the release of bioactive peptides which have been reviewed in many articles.[11,16,17,21,22]

Sleep enhancing and anxiolytic-like properties have been attributed to milk particularly in newborns[23,24] and it is suggested that having milk at night is a promising natural sleep aid.[25] Indeed, milk is rich in sleep-promoting components such as tryptophan and melatonin.[25] In addition, the soothing effect of milk might be attributed to peptide fragments released after gastrointestinal digestion of milk proteins. Hence, when bovine milk proteins were investigated for the speculated sedative and tranquilizing properties of milk, different peptides, *i.e.*, α-casozepine (α-CZP), β-lactotensin (β-LT) and wheylin-1 and -2, with anxiolytic and anti-stress properties were identified. These are discussed in detail in the following section.

2.1. α-Casozepine and its structure

α-CZP is an anxiolytic decapeptide that corresponds to residues 91–100 (YLGYLEQLLR) of bovine α_{s1}-casein (α_{s1}-CN). It is present in α_{s1}-CN tryptic hydrolysate (CTH) as an active ingredient and has been found to display anxiolytic-like, anticonvulsant and anti-stress properties in humans, rodents and other animal species (Tables 1 and 2). The conformational studies conducted in pure water and/or dimethylsulfoxide by two-dimensional-NMR and restrained molecular dynamics indicate the structural flexibility of α-CZP.[26] Nevertheless, α-CZP displayed a defined structure in a micellar medium of water and sodium dodecyl sulfate (SDS). Under these conditions, the ^{3}GYLEQLL^{9} sequence of α-CZP adopts a helicoidal structure, *i.e.* a 3_{10}-helix initiated and terminated by an α-turn. This helical structure is further stabilized by ionic interactions between the guanidinium function of the C-terminal arginine residue and (i) the α-carboxylate function of this residue and (ii) the γ-carboxylate function of the glutamic residue. Moreover, the arrangement of the hydrophobic residues of α-CZP on one face of the helix and hydrophilic residues on the other face confers it an amphipathic character. The spatial structure, thus adopted by α-CZP in the SDS/water micellar medium, revealed some conformational similarities to the BDZ nitrazepam. Indeed, a comparable distance has been shown between the centers of the aromatic rings of the two residues of tyrosine of α-CZP and the two aromatic rings of crystallized nitrazepam. This suggested that the tyrosyl residues of α-CZP adopt, under certain conditions, a relative disposition similar to that of the aromatic rings of nitrazepam that may allow the recognition of α-CZP by the BDZ site of γ-amino-butyric acid type A ($GABA_A$) receptors. This also emphasizes that the integrity of α-CZP (importance of the first residue of tyrosine and the final residue of arginine) could be crucial for its pharmacological activity.[26]

2.1.1. Biological activities of casein hydrolysate containing α-CZP and purified α-CZP.

2.1.1.1. Studies in rodents

Numerous preclinical studies indicate that pure α-CZP, pure CTH obtained under laboratory conditions, or industrial CTH (i-CTH marketed under the name “Lactium®”) plays an important role in improving responses to anxiety and stress conditions (Table 1). In rodents, anxiolytic-like activity was mostly demonstrated in two ethological models of anxiety: conditioned defensive burying paradigm (CDB) and elevated plus-maze test (EPM). The CDB paradigm is a test that exploits the propensity of rats to bury objects of aversive stimulation and is used to study the neuronal mechanisms underlying anxiety regulation,[27] whereas the EPM model exploits the conflict between exploration and fear of open spaces in rodents and belongs to the group of unconditioned anxiety paradigms for developing putative anxiolytic compounds.[28,29] Both tests are used to evaluate the anxiolytic-like activity of α-CZP (intraperitoneal (i.p.) and/or oral route), CTH (i.p. route) and i-CTH (oral route), whereas the light/dark box (LDB) test is only carried out for α-CZP (i.p. route).[30–33]

Miclo and his coworkers (2001) reported for the first time that i.p. administration of CTH (3 mg kg^{-1}) in rats showed an anxiolytic-like activity in the EPM test, as reflected by an increase in both the time the rats spent in open arms, the most anxiogenic part of the plus maze, and the number of open arm entries.[30] Identical effects were obtained in the EPM test when pure α-CZP (1 mg kg^{-1}) was injected in rats by the i.p. route.[32] Likewise, an increase in LDB test parameters (the time spent as well as the number of rears in

the lighted box) was observed in mice after i.p. injection of α-CZP (1 mg kg^{-1}).[34] In the CDB paradigm, i.p. administration of α-CZP or CTH (0.4 and 3 mg kg^{-1}, respectively) significantly decreased the anxiety parameters like probe burying duration, the percentage of approaches towards the probe followed by retreats and the number of heads stretching towards the probe in rats after receiving an electrical shock.[30] Besides i.p. administration, certain reports illustrate that i-CTH has effectively reduced the anxiety symptoms when taken orally in higher quantity than by the i.p. route. According to Violle and colleagues (2006), the minimal recommended dose, which significantly reduced the anxiety levels in rats in the CDB test after *per os* (p.o.) administration of i-CTH, is 15 mg kg^{-1}. They reported that in the EPM test and CDB paradigm (Table 1), the oral intake of i-CTH at this dose efficiently induced behavioral changes in rats.[31] Similarly, in another study the CDB behavioral test was conducted in rats and the anxiolytic-like action of orally taken i-CTH (15 mg kg^{-1}) was observed even after 7 days twice daily treatment (Table 1). These studies signify a potent anxiolytic-like profile of i-CTH after oral intake similar to diazepam without visible side effects such as risk-taking behavior and lack of an induced tolerance that can be observed after subchronic oral intake of diazepam at a dose of 3 mg kg^{-1}. Furthermore, the rats did not show any symptoms of anterograde amnesia or addiction to i-CTH after p.o. administration of this hydrolysate at a higher dose (150 mg kg^{-1}) in the passive avoidance test and the conditioned place preference test, respectively.[31,35] Hence, it can be said that i-CTH does not show the side effects commonly observed in the therapeutic use of BDZs. BDZs are not only anxiolytics, but also oppose the effects of pentylenetetrazol, a molecule that induces epileptic symptoms by acting on $GABA_A$ receptors, in rodents and fight against generalized myoclonic seizures in humans. They are also effective against absence seizures.[36] When administered by the i.p. route (3 mg kg^{-1}), the anxiolytic-like CTH also reduced the epileptic symptoms (Table 1) induced by pentylenetetrazol in rats.[30] The pharmacological profile and the structure of α-CZP in micellar medium and the pharmacological profile of CTH or i-CTH containing this peptide suggest that the $GABA_A$ receptor could be the target of the molecule. However, this hypothesis has not yet been formally demonstrated. The protein expression of the β_1 subunit but not that of the α_1 subunit of the $GABA_A$ receptor was found to be increased in the hypothalamus of rats orally treated with 150–300 mg kg^{-1} of i-CTH.[37] The modification of the subunit expression of the $GABA_A$ receptor does not, however, involve a direct action on this receptor.

2.1.1.2. Studies in other animals

Many promising preclinical reports indicate the routine use of i-CTH for the treatment and/or prevention of anxious disorders in animals other than rodents (Table 2). Beata *et al.* (2007) have shown in a double-blind placebo-controlled trial that i-CTH played a significant role in the management of cats that display anxiety in socially stressful conditions. Cats treated with i-CTH (15 mg kg^{-1}, p.o.) sought contact with both familiar and non-familiar people and showed an improvement in fearful behavior and in the associated autonomic signs (evaluated from the Cat Emotional Scale).[38] Similarly, in a randomized double-blind placebo-controlled trial, the cats were given different diets twice daily for 8 weeks to determine their response to stressors like veterinary examination and blood sampling. The cats fed on a study diet containing tryptophan and supplemented with i-CTH showed a significantly higher ratio of plasma tryptophan to large neutral amino acids (LNAA; sum of tyrosine, phenylalanine, valine, leucine and isoleucine) as well as decreased urinary cortisol (physiological indicator of stress) levels compared to the control diet group. However, no change in the plasma cortisol concentration was observed after exposure to the stressors studied.[39]

When dogs are exposed to excessive chronic stress, behavioral disorders such as licking of the paws and fear reactions can occur leading to responses like aggression, barking or tendency to destroy. These abnormalities are the most common cause of abandonment by dog owners. Psychotropic drugs can be used, but are often rejected by the owners and, therefore, they preferred dietary supplements when this alternative is possible. In a double-blind randomized comparative trial in dogs suffering from anxiety-related conditions, the oral intake of i-CTH at a dose of 15 mg per kg per day markedly reduced anxiety symptoms (Table 2) and the observed effects were similar to those of a treatment with selegiline hydrochloride, a monoamine oxidase inhibitor used in dogs for the treatment of behavioral disorders of emotional origin.[40] Similarly, in a randomized double-blind placebo-controlled trial conducted in female dogs, the anxious dogs fed on a diet containing i-CTH exhibited an improved behavioral score (assessed from the score of Reactivity Evaluation Form) in terms of decreased exploratory behavior and better orientation toward the environment compared to the anxious dogs fed on the placebo diet. The supplemented diet does not alter the anxiety level in non-anxious animals while it decreases this level in anxious animals. Besides behavioral changes, the i-CTH-supplemented diet also significantly decreased the plasma cortisol levels in anxious dogs.[41] Likewise, in a single-blind crossover trial, after exposure to a stressful condition (visit to a veterinary clinic for toenail clipping), a significant decrease in the increase of urinary cortisol to creatinine ratio was observed in dogs fed on a diet containing tryptophan and supplemented with i-CTH while no such effects were observed in dogs fed on the control diet. Furthermore, the anxious dogs administered with the supplemented diet also displayed improved behavioral scores (evaluated from the Canine Behavioral Assessment and Research Questionnaire) as reflected by reduced stranger-directed fear, nonsocial fear and touch sensitivity.[42] The anxiolytic action may be attributed to i-CTH as Bosch and colleagues (2007) showed that tryptophan alone does not change the anxious behavior in dogs.[43]

Horses may also suffer from physical or psychological stress induced by equestrian activities, fear or anxiety due to modifications of their environment, social separation, transport and pain or discomfort.[44] In a blind study, the calming effect of i-CTH on ponies has also been reported during their transition from semi-feral conditions to domestic facility for introduction to basic ground handlings. The ponies treated with i-CTH (1000 mg per day, p.o., for a weight between 160 and 205 kg leading to a dose between 4.9 and 6.2 mg kg^{-1}) showed a better overall performance than their matched counterparts fed on the control supplement (oat flour), as determined from their ranking based on objective

measures (1 = best of the 6 ponies, 6 = poorest). The mean of sums of the ranks for all the fourteen training and/or handling sessions for i-CTH fed ponies was 35.2 compared with 62.8 for control ponies ($p < 0.05$), indicating a significantly better performance of the i-CTH treated ponies. At 6 weeks after the 2-week training period, orally i-CTH-treated ponies again showed a better score upon reassessment of the seven specific skills including approach and catch at pasture, leading, lifting/picking out feet, intranasal application and clipper, and cross-tie.[45] Likewise, i-CTH supplementation modestly improved the mares' compliance and comfort to mildly aversive routine management and healthcare procedures in a 5-day blind study. The mares treated with i-CTH (2000 mg per day, p.o., for a weight ranging from 450 to 600 kg) showed improved compliance and comfort score for different aversions such as examination room entry, eye medication, intranasal treatment, jugular stick, lip twitch application and trailer loading compared to their matched counterparts fed on the control supplement, *i.e.* oat flour.[46]

2.1.1.3. Studies in humans.

In addition to animals, i-CTH has also shown anxiolytic-like effects on humans. Messaoudi *et al.* (2005) subjected 42 healthy male volunteers to mental and physical stress conditions (the Stroop test and Cold pressor test, respectively) in a double-blind placebo-controlled trial (placebo was a skimmed milk powder) and examined the influence of i-CTH ingestion (3 doses of 400 mg at a 12 h interval, the last one 2 h before the beginning of the test) on hemodynamic parameters such as systolic and diastolic blood pressure together with the heart rate. They reported a significant improvement in stress-related physiological variables (Table 2) in the treated subjects compared to the placebo group.[47] Similarly, Lanoir and colleagues (2002) also reported a significantly lower increase in the systolic blood pressure of highly stressed responder women after 30 days of treatment in a randomly assigned double-blind trial.[48] Moreover, a double-blind randomized crossover placebo-controlled trial was conducted in 63 female volunteers experiencing at least one of the stress-related disorders such as anxiety, sleep or general fatigue. The results showed that 30-day ingestion of encapsulated i-CTH at a dose of 150 mg per day alleviated certain stress symptoms in women. The hydrolysate was particularly effective at improving cardiovascular, digestive, intellectual, emotional and social problems in subjects who demonstrated the highest initial intensities for their major stress symptoms.[49] Similarly, in an open-label study with 100 outpatients undergoing psychiatric consultation in private clinics for anxiety/sleep disorders, the subjects were given a dietary supplement containing α-CZP at a dose of 300 mg per day. After 4-week treatment, a significant decrease in anxiety/sleep symptoms was reported as observed from the comparison of mean scores of all rating scales (the Hamilton Anxiety scale, the Insomnia Severity Scale, the Clinical Global Impression Scale and the Dosage Record and Treatment Emergent Symptom Scale).[50] Recently, it has been reported that in professional participants twice daily intake over 12 weeks of a dietary supplement containing i-CTH, taurine, *Eleutherococcus senticosus* and a proprietary melon juice concentrate significantly improved the symptoms of burnout syndrome (work-related crisis) in a double-blind randomized placebo-controlled trial. The men and women ingesting the dietary supplement showed significant improvements in scores for different burnout evaluation tools like (i) BMS-10 (Burnout Measure Short version), (ii) MBI-HSS (Maslach Burnout Inventory-Human Services Survey) fatigue, (iii) Beck Depression Inventory, (iv) MBI-HSS depersonalization and (v) task management (Table 2), whereas for placebo-treated subjects only scores for the first three evaluation tools were improved. The authors attributed this improvement to α-CZP present in i-CTH of dietary supplement.[51] In the studies conducted in human beings, it is difficult to determine the optimal dose to be administered in order to obtain sufficient anxiolytic effects. In fact, the doses administered or the administration schedules were different and the weight of individuals was not recorded. So, the question remains whether i-CTH is more effective in women or this effectiveness is due to their lower average weight compared to men.

2.1.1.4. Effect on sleep. The role of i-CTH in the management of stress-induced sleep disorders has been supported by many studies conducted in both rodents and humans. Guesdon *et al.* (2006) pointed out that the oral intake of i-CTH (15 mg per kg per day during 8 days) prevented sleep disturbances in rats subjected to mild chronic stress. The total sleep duration remained unchanged during the first 2 days of the mild stress period in the rats ingesting i-CTH but decreased significantly in the control group (feeding on total bovine milk proteins). Maintenance of the total sleep duration in rats treated with i-CTH is attributed to a stable duration of slow wave sleep with a slight increase in paradoxical sleep duration.[52] In a recent study, the sleep-promoting effect of i-CTH has been shown by the pentobarbital-induced sleeping test and electrophysiological (EEG) monitoring (a parameter to evaluate and diagnose sleep-related problems/disorders). When i-CTH (150 mg kg^{-1}) was administered orally, it did not only significantly enhanced the duration of the sleep induced by pentobarbital sodium in mice, but also promoted the slow-wave (delta) EEG activity in rats.[53] In another pentobarbital sodium-induced sleep study, the oral intake of i-CTH at 120 or 240 mg kg^{-1} increased the sleep duration in mice. When given at 300 mg kg^{-1}, it enhanced the total sleep time and reduced about 50% sleep–wake cycles in rats.[37] It was also reported in humans that chronic administration of one capsule per day of i-CTH, corresponding to a dose of 2.70 mg of α-CZP (150 mg kg^{-1} i-CTH), in a double-blind parallel trial, markedly improved the sleep quality of Japanese men and women who had a full-time day-time job and complained of insomnia. The subjects treated with i-CTH showed a significant decrease in sleep latency and daytime dysfunction (assessed from the Japanese Pittsburg Sleep Quality Index) compared to placebo subjects (mixture of dextrin, gelatin and titanium dioxide).[54] Similarly, patients suffering from sleep disorders were administered a dietary supplement (300 mg per day) containing α-CZP for 4 weeks and about half of the patients reported a pro-hypnotic effect of this dietary supplement.[50] Recently, in a 12-week double-blind randomized crossover trial, i-CTH administration improved the sleep quality in individuals having varying degrees of sleep disturbance compared to the placebo group (Table 2). The beneficial effects were even better with a prolonged intake period of i-CTH.[55] In another double-blind randomized trial, daily oral intake of a supplement (150 mg i-CTH and 50 mg L-theanine) for 4 weeks significantly improved the sleep

latency score, sleep disturbance score and daytime dysfunction score in the intervention group. Moreover, supplement administration also reduced the anxiety, depression and stress levels in the same group.[56] All the studies described above ultimately signify the role of i-CTH in improving insomnia-like sleep disorders.

2.1.2. Metabolism of α-CZP: potential anxiolytic activity of shorter fragments.

In vitro simulated digestion of α-CZP by pepsin, trypsin, α-chymotrypsin and Corolase PP™ (a mixture of pancreatic enzymes) indicated that it was hydrolysed to shorter N-terminal fragments in a way depending on the nature of the enzymes used. For instance, half of the α-CZP was found to be intact after 25 min (the beginning time of the gastric emptying of a solid meal) exposure to pepsin at pH 2.0 or 4.0, while little hydrolysis was observed when incubated with α-chymotrypsin. The main products obtained after hydrolysis by pepsin followed by Corolase PP™ corresponds to sequences 91–97 and 91–95 of α-CZP. Surprisingly, these two fragments also displayed an anxiolytic-like profile after i.p. administration. The fragment 91–97 showed anxiolytic-like activity[32] in three behavioral tests in rats (0.5 mg kg^{-1} or 0.7 mg kg^{-1} according to the test; Table 1) and the fragment 91–95 in mice after i.p. administration at 0.5 mg kg^{-1} in the LDB test.[57]

It has been reported that both α-CZP and the fragment 91–97 are also hydrolysed by the brush border membrane peptidases of the human colon carcinoma cell line Caco-2. Only α-CZP was hydrolysed to N-terminal fragments by carboxypeptidases of these cells but this effect was strongly counteracted in the presence of bile salts, a model approaching physiological processes. Aminopeptidase and dipeptidyl peptidase activities were observed in both peptides but the rate of hydrolysis of the fragment 91–97 varied highly depending on the nature of bile salts leading to a greater resistance of this peptide towards hydrolysis under certain conditions. Moreover, the transfer of these peptides across the Caco-2 monolayer cells increased significantly in the presence of bile salts.[58]

Apart from α-CZP and its shorter fragments YLGYLEQ and YLGYL, two other shorter peptides YL and YLG also displayed anxiolytic-like activity in behavioral tests in mice. The dipeptide YL exhibited anxiolytic-like activity when injected by the i.p. route (0.1–1 mg kg^{-1}) 30 min before the EPM test as determined by the increased percentage of time spent in and the number of visits in open arms. This anxiolytic-like activity of YL was comparable to diazepam when injected at the same dose (1 mg kg^{-1}). In the open field test (exploiting the conflict between exploration and innate fear that rodents have of the central area of a novel or brightly lit open field), i.p. administration (0.1 mg kg^{-1}) of YL increased the time spent in and the number of visits in the center circle without affecting the locomotor activity. Similar results were obtained in the EPM test when YL was taken orally or by the intracerebroventricular (i.c.v.) route at a dose of 0.3–3 mg kg^{-1} and 0.1 nmol per mouse (29.4 ng per mouse), respectively.[59] Similarly, the tripeptide YLG corresponding to the fragment 91–93 of α_{s1}-CN also exhibited anxiolytic-like activity in the EPM and open field tests in mice of two strains. In the EPM test, i.p. administration of YLG (1–3 mg kg^{-1}) alone or pepsin-pancreatin digest of α_s-CN (0.1–1 g kg^{-1}; containing 0.27–2.7 mg kg^{-1} YLG) significantly increased the percentage of time spent in and visits to open arms. Identical effects were observed when YLG was administered orally (1.2 mg kg^{-1}). Likewise, the percentage of time spent in and visits to open circles were increased significantly in the open field test after i.p. administration (0.1 mg kg^{-1}) of the same peptide.[33]

2.1.3. Mode of action of α-CZP and its safety profile.

The binding sites of most anxiolytics are located in the central nervous system (CNS) and are unevenly distributed in different brain regions such as amygdala, hippocampal formation and cerebral cortex. In addition, some neurotransmitters are also associated with anxiety regulation and this regulation includes $GABA_A$ receptors, ligand-gated chloride channels, and $5HT_{1A}$ receptors of the serotoninergic system.[60–62] It is commonly accepted that BDZs bind allosterically to the $GABA_A$ receptor and potentiate the chloride channel opening by γ-amino-butyric acid (GABA: negative mediator of neurotransmission) and consequently enhance the inhibitory effect of GABA.[63]

The mode of action of α-CZP is not well known. However, it is presumed to be central as α-CZP and i-CTH displayed anxiolytic-like activity *in vivo* after i.p. administration. A study conducted by Miclo and colleagues (2001) reported the affinity of α-CZP for the BDZ site of the $GABA_A$ receptor as it displaces the tritiated flunitrazepam from this site *in vitro* with a half maximal inhibitory concentration (IC_{50}) of 88 μM. Although its affinity for the BDZ site of $GABA_A$ receptors is 10

000 times lower than diazepam (IC_{50} = 8.2 nM for diazepam), the *in vivo* efficacy of α-CZP is comparable to that of diazepam.[30] Despite this difference in IC_{50}, the effect of α-CZP *via* the BDZ site of the $GABA_A$ receptor cannot be excluded. Indeed, there are 19 different subtypes of $GABA_A$ receptors, which differ in abundance throughout the CNS.[64] In addition, BDZ affinity varies from one subtype to other. For example, flunitrazepam is a non-selective BDZ which binds to $GABA_A$ receptors composed of, in addition to β and γ subunits, α_1, α_2, α_3 or α_5 subunits. The affinity of flunitrazepam was even highlighted on receptors possessing an α_4 subunit associated with the β3 subunit, while the $GABA_A$ receptors possessing an α_4 or α_6 subunit have been reported to be insensitive to diazepam.[65] Therefore, more than 90% of the $GABA_A$ receptors of the CNS are targeted by flunitrazepam. Hence, two hypotheses can be proposed to explain an apparent lower affinity of α-CZP for $GABA_A$ receptors. Firstly, if α-CZP targets a single $GABA_A$ receptor subtype, it would only displace flunitrazepam fixed on this subtype and not that fixed on all the other subtypes, consequently leading to an overestimated IC_{50}. Secondly, if flunitrazepam has a lower affinity for the $GABA_A$ receptor subtype(s) targeted by α-CZP, it would occupy only a part of the binding sites of α-CZP leading once again to an overestimated IC_{50} (Fig. 2). It can be noted that ocinaplon, a pyrazolopyrimidine, displaces flunitrazepam from the $GABA_A$ receptors of the rat cerebral cortex with a low affinity ($IC_{50} \approx 2$ μM) but is sensitive to

flumazenil, a benzodiazepine antagonist, in the EPM test in rats.[66] $GABA_A$ hypothesis was reinforced by another *in vitro* study on human neuroblastoma cells. Indeed, i-CTH potentiated the GABAergic neurotransmission, notably through the $GABA_A$ receptor–chloride ion channel complex, which was blocked by co-administration of the $GABA_A$ receptor antagonist bicuculline.[53] A recent study conducted in mice facing an anxiety-inducing situation showed that α-CZP (1 mg kg^{-1}, i.p.) administration modulated the neuronal activity (c-Fos expression) in different brain regions involved in anxiety regulation. α-CZP decreased c-Fos expression in the hippocampal formation, accumbens nucleus, bed nucleus of the stria terminalis and nuclei of the hypothalamus, and increased this expression in the raphe magnus nucleus and in the basolateral, basomedial, medial and posterolateral cortical nuclei of the amygdala.[34,57] This pointed out the differences with diazepam since this BDZ exhibited no effect on global c-Fos expression in the amygdala but in the prefrontal cortex. These differences in the brain modulation pattern may explain the absence of the side effects of α-CZP in contrast to BDZs. Like α-CZP, the fragment 91–95 had a similar effect on c-Fos expression except in the different nuclei of the amygdala, which might indicate a different mode of action of the shorter fragment.[57] These studies thus confirm the central mode of action of the peptide α-CZP and one of its fragments.

α-CZP does not need to be modified in the gastrointestinal tract since i.p. injection leads to an anxiolytic-like activity. However, it is still intriguing that whether the whole peptide sequence of α-CZP or shorter sequences are responsible for its central mode of action after oral administration. α-CZP was hydrolysed to shorter peptide fragments after exposure to gastrointestinal enzymes.[32] It should remain intact during gastrointestinal passage to display its activity, as it seems that both N- and C-terminal residues are important to obtain a structure able to interact with the BDZ site of the $GABA_A$ receptor. Surprisingly, the shorter peptides YLGYLEQ and YLGYL also displayed anxiolytic-like activity in the EPM, CDB or LDB tests in rats or mice. Thus, it can be proposed that these peptide fragments participated in the *in vivo* activity of α-CZP with a mode of action that might be slightly different as was shown for YLGYL.[57] Knowing the residues involved in maintaining the structure of α-CZP in micellar medium, and especially the C-terminal arginine residue, the anxiolytic activity of a shorter peptide was an unexpected result. Nevertheless, this result suggests that the arginine residue, present in the decapeptide, may lead to the active structure despite the steric constraints imposed by the additional residues (^{98}LLR^{100}) absent from the peptides YLGYLEQ and YLGYL. The removal of some carboxy-terminal residues of α-CZP may remove these constraints and allow the shorter peptides to adopt an active form. Moreover, two shorter peptides YL and YLG, corresponding to the N-terminal sequence of α-CZP, also displayed anxiolytic-like activity *in vivo*. The anxiolytic-like activity of YL and YLG was inhibited in the presence of antagonists of 5-HT_{1A}, D1 and $GABA_A$ receptors (associated with anxiolytic-like activity in animals and humans) but both peptides displayed a lack of affinity for all these receptors. This suggests that the anxiolytic-like activity of YL and YLG may be mediated *via* an increase in the release of endogenous neurotransmitters like serotonin, dopamine and GABA, and *via* the activation of 5-HT_{1A}, D1 and $GABA_A$ receptors.[33,59] The di- and tripeptides that might be formed from α-CZP or its shorter fragments (YLGYLEQ and YLGYL) may therefore have a mode of action totally different from that of these peptides.

The anxiolytic-like activity of YL leads to a notable remark. In proteins, the leucine residue has a frequency of about 7.5 to 8.9% and the tyrosine residue has a frequency of about 3.0 to 3.4% depending on the protein set considered.[67,68] The sequence of residues in a protein is not due to a simple chance since the frequencies calculated for the dipeptides can deviate from the calculated frequencies of various residues. Thus, the dipeptide YL has a frequency in proteins equal to 0.28%.[69] The frequency of the dipeptide YL indicates that this is not a rare sequence and therefore proteins containing this sequence can be consumed daily. If all potential dipeptides YL could be released by the proteolytic enzymes of the gastrointestinal tract, their amount could reach about 225 mg per day (3.75 mg kg^{-1}) for an adult consumer. This would mean that the consumer should be in postprandial anxiolysis since the simple YL sequence triggers an anxiolytic action in mice.

The side effects such as induced tolerance, anterograde amnesia, risk taking behavior, sedation and memory loss, which are usually associated with the chronic use of BDZs, have not yet been reported for i-CTH. These differences in the pharmacological profile might be related, as mentioned above, to the different neuronal modulation that was observed for α-CZP and diazepam. Moreover, α-CZP is given a GRAS status by the US FDA (FDA-21 CFR §184.1553; NDI #rpt242) and a claim by the Australian Therapeutic Goods Administration (TGA). It has also received a health claim authorization from the DGCCRF (French General Directorate for Competition Policy, Consumer Affairs and Fraud Control) approved by the AFSSA (French Health and Food Safety Agency). (i) The safety profile and claims accorded to α-CZP, (ii) its increased ability to cross the intestinal barrier under certain conditions and (iii) its biological efficiency validate the suitability of this peptide for functionalization. Therefore, pure α-CZP or that present in i-CTH or its shorter fragments 91–97 and 91–95 could be suitable candidates for the development of functional foods, especially fermented dairy products.

2.2. β-Lactotensin

β-Lactotensin (β-LT) is a bioactive tetrapeptide (HIRL) which corresponds to the residues 146–149 of bovine whey protein β-lactoglobulin (β-Lg). It was isolated from the chymotryptic digest of β-Lg and its ability to induce the contraction of longitudinal ileum muscles was first demonstrated in male Hartley Guinea pigs by Yamauchi and colleagues (2003).[70] Although β-LT has homology with neurotensin and is characterized as the natural agonist of the neurotensin receptor subtype 2 (NTS_2), its ileum-contracting activity is 1000 times lower than that of neurotensin.[70] Neurotensin is an endogenous tridecapeptide originally isolated from calf hypothalamus and is widely distributed in the CNS as well as gastrointestinal and cardiovascular systems.[71] It functions as a neurotransmitter or a neuromodulator in the CNS and as a local (paracrine and circulation) hormone in the periphery,

mainly the gastrointestinal tract.[72,73] There are three subtypes of neurotensin receptors, *i.e.* NTS_1, NTS_2 and NTS_3. Both NTS_1 and NTS_2 subtypes belong to the guanine nucleotide-binding protein (G protein)-coupled family of receptors with seven transmembrane domains, while NTS_3, a structurally different subtype, is identical to gp95-sortilin protein having a single-transmembrane domain. The NTS_1 receptor is a high-affinity site for neurotensin (sub nanomolar range) while the NTS_2 receptor is a low-affinity site (nanomolar range).[71,73] The NTS_1 receptor is involved in most intestinal responses to neurotensin, while the NTS_2 receptor is mainly expressed in the brain and organs outside the gastrointestinal zone.[74]

There are some studies which indicate the potential physiological effects of β-LT after interaction with neurotensin (particularly NTS_2) and dopamine receptors among others. It has been shown to display hypertensive, hypocholesterolemic, antinociceptive, and anorexigenic activities as well as anxiolytic-like activities in rats and mice (Table 3). In the EPM test, β-LT dose-dependently exhibited anxiolytic-like activity following i.p. administration of 1–3 mg kg^{-1} in C57BL/6 mice and 10 mg kg^{-1} i.p. or 3–10 mg kg^{-1} oral administration in ddY mice. The β-LT treated mice showed a significant increase in the percentage of time spent in and the number of visits to the open arms in the EPM test compared to the saline treated group. Similarly, the increased percentage of time spent in the circle (central area) was reported in the β-LT treated mice (3 mg kg^{-1}, i.p.) in the open field test, whereas no significant difference was observed in their locomotor activity. The anxiolytic-like activity of β-LT is mediated by the NTS_2 receptor because the activity was inhibited in the presence of the NTS_2 receptor antagonist levocabastine (0.05 mg kg^{-1}, i.p.). Moreover, no such effects were described in *ntsr2*-knockout mice. The activation of the NTS_2 receptor after β-LT intake was also evident from the increased intracellular Ca^{2+} flux in the glial cells of wild-type mice but not in *ntsr2*-knockout mice. The anxiolytic-like activity of β-LT was also mediated by the dopamine D_1 receptor, as the D_1 receptor antagonist SCH23390 (10 μg kg^{-1}, i.p.) blocked the anxiolytic-like activity. It is assumed that β-LT exhibits anxiolytic-like activity by stimulating the D_1 receptor downstream of the NTS_2 receptor without involving the D_2 receptor.[75]

β-LT also displayed anti-stress activity *via* the NTS_2 receptor in the mice subjected to acute restraint-induced stress. β-LT treated mice (30 mg kg^{-1}, i.p.) showed significantly improved stress-induced behaviors like an increased number of head tips and shortened latency to the first head-dip compared to the saline control group in the hole-board test. In addition, β-LT also promoted the extinction of fear memory in the mice subjected to considerably potent electric shock as reflected by the lessened freezing responses in the fear conditioning test.[76] It was further described that the i.c.v. injection (60 nmol per mouse *i.e.* about 32.3 μg per mouse) as well as oral ingestion (300–500 mg kg^{-1}) of β-LT increased memory consolidation in mice in the step-through-type inhibitory avoidance test by modulating the dopamine system. Nevertheless, administration of β-LT just after conditioning improved the step-through latency but remained ineffective if administered 2 h after the conditioning. The memory-enhancing activity of β-LT was impeded by the dopamine D_2 receptor antagonist raclopride (0.15 μg per mouse, i.c.v.) but not by the D_1 receptor antagonist SCH23390 (0.3 μg per mouse, i.c.v.) suggesting that enhanced memory consolidation was mediated by dopamine release followed by activation of the D_2 receptor, but not *via* the direct interaction as the affinity of β-LT for this receptor is of the order of 100 μM.[77]

2.3. Wheylin-1 and 2

Wheylin-1, a dipeptide (MH), and wheylin-2, a tripeptide (MKG), corresponding to the residues 145–146 and 7–9, respectively, of bovine β-Lg are bioactive peptides derived from the thermolysin digest of this whey protein. Their anxiolytic-like activity was first demonstrated in behavioral paradigms in mice. In the EPM test, i.p. administration of wheylin-1 (0.3–1 mg kg^{-1}) or wheylin-2 (3 mg kg^{-1}) 30 min before the test significantly increased the percentage of time spent in open arms of the maze. Wheylin-1 is a more potent anxiolytic than wheylin-2, since the minimum effective dose of the latter is 10 times more than the former. Similar effects in the same test were reported after oral (1 mg kg^{-1}) or i.c.v. (1 nmol per mouse; about 0.29 μg per mouse) administration of wheylin-1. In the open field test, wheylin-1 (0.1 mg kg^{-1}, i.p.) significantly increased the percentage of time spent in and the number of visits to the center circle without affecting the locomotor activity. However, the anxiolytic-like activity of wheylin-1 (1 mg kg^{-1}, i.p.) was inhibited in the presence of bicuculline (5 mg kg^{-1}, i.p.). Bicuculline is a GABA antagonist that binds directly to the site of this neurotransmitter present on the $GABA_A$ receptor. Although this molecule can also lead to conformational changes in the receptor, the primary action of bicuculline is to occupy the GABA binding site.[78] However, wheylin-1 showed a negligible affinity for the BZD and GABA sites of the $GABA_A$ receptor at a concentration of 100 μM. Moreover, wheylin-1 did not act through the opioid system. This suggests that the anxiolytic-like activity of wheylin-1 might be mediated *via* activation of the $GABA_A$ receptor system.[79] The frequency of the MH sequence in proteins of 614 eukaryote proteomes is 0.052%.[69] This could lead to a daily consumption of 40 mg of the dipeptide by an adult if the proteolytic enzymes of the gastrointestinal tract release all the MH sequences from food proteins. Consequently, a postprandial anxiolysis might then be considered in humans but only if the peptide is active in them and is able to reach its biological target(s).

3. Peptides from other food proteins

Besides α-CZP and β-LT, the most studied anxiolytic peptides of milk origin, and wheylins, peptides from other food proteins also displayed anxiolytic-like activities which are discussed in the following sections

3.1. Rubiscolin-6 and rubimetide from rubisco

Rubiscolin-6 (YPLDLF) is a bioactive hexapeptide (103–108) isolated from a gastrointestinal enzymatic digest of the large

subunit of D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; E.C. 4.1.1.39) of spinach leaves. Rubisco is the key enzyme for carbon dioxide fixation and photorespiration. It is known to be the most abundant protein on the Earth because of its ubiquitous existence in substantial amounts (~30–50% of soluble proteins) in plants.[80] Rubiscolin-6 is implicated in the modulation of different physiological actions such as analgesic, antinociceptive, orexigenic and anxiolytic-like activities after central or peripheral administration.[80–82]

The administration of rubiscolin-6 in mice by the i.p. or p.o. route at a dose of 10 mg kg^{-1} (13 μmol kg^{-1}) or 100 mg kg^{-1} (130 μmol kg^{-1}), respectively, notably increased the percentage of the time spent in and the number of entries to the open arms in the EPM test. It was revealed that the anxiolytic-like activity of rubiscolin-6 after administration by both routes was blocked by the δ opioid receptor antagonist naltrindole (1 mg kg^{-1}, s.c.), the dopamine D_1 receptor antagonist SCH23390 (30 mg kg^{-1}, i.p.), and the σ_1 receptor antagonist BMY14802 (0.5 mg kg^{-1}, i.p.) or BD1047 (10 mg kg^{-1}, i.p.). The inhibition studies showed that the anxiolytic-like activity of rubiscolin-6 was mediated by both dopamine D_1 and σ_1 receptors downstream of the δ opioid receptor, although rubiscolin-6 has no affinity for both dopamine D_1 and σ_1 receptors. Indeed, the δ opioid receptor stimulated by rubiscolin-6 activated further the σ_1 receptor-dependent mechanism, which in turn induced dopamine release and stimulation of the D_1 receptor resulting in an anxiolytic-like effect.[83] Furthermore, rubiscolin-6 has also been shown to enhance the memory consolidation in a step-through type passive avoidance test in mice. The step-through latency was significantly improved after i.c.v. (3 nmol per mouse; 2.3 μg per mouse) and oral (100 mg kg^{-1}) intake of rubiscolin-6 in the conditioned mice.[84] The enhanced memory consolidation activity was mediated by the interaction of rubiscolin-6 with the δ opioid receptor. Indeed, this effect was also hindered by naltrindole (1 mg kg^{-1}, s.c.). A link between opiate receptors and anxiety is established since knockout mice for the gene encoding the δ receptor showed an anxiogenic and depressive response in different models. In contrast, the knockout animals for the gene encoding the opiate κ receptor showed no particular phenotype.[85]

Rubimetide is another anxiolytic peptide (MRW) that was isolated from the pepsin-pancreatin digest of a large chain of spinach Rubisco and corresponds to the residues 212–214 of this protein. Similar to rubiscolin-6, this tripeptide has shown anxiolytic-like activity in mice in the EPM test (increased percentage of the time spent in and the number of entries to the open arms) after oral (1 mg kg^{-1}; 2 μmol kg^{-1}) or i.p. (0.1 mg kg^{-1}) administration. Despite the no measurable affinity of rubimetide for the prostaglandin DP_1 receptor at 100 μM, its anxiolytic-like activity was hindered in the presence of the DP_1 receptor antagonist BW A868C (60 μg kg^{-1}, i.p.). It means that the rubimetide-induced anxiolytic-like activity was rather mediated by PGD2-dependent DP_1 receptor activation instead of σ and dopamine D_1 receptors as in the case of rubiscolin-6.[86] A recent study also supports the involvement of these receptors along with formyl peptide receptors (FPR) in the anxiolytic-like activity of rubimetide. FPR are a small group of seven-transmembrane domain, G protein-coupled receptors that are mainly expressed by mammalian phagocytic leukocytes and are known to be important in host defense and inflammation.[87] The anxiolytic-like activity of rubimetide was found to be mediated by interacting with FPR subtype FPR2 (despite of low affinity for FPR2, K_i = 266 μM) as this activity was blocked in the presence of the FPR2 antagonist WRW4 (10 mg kg^{-1}, i.p.). In addition, this activity was also blocked by BW A868C (1.6 nmol per mouse, i.c.v), SCH58261 (1 mg kg^{-1}, i.p.) and bicuculline (5 mg kg^{-1}, i.p.), the antagonists of the DP_1 receptor, adenosine A_{2A} and the GABA site of $GABA_A$ receptors, respectively. This shows that the anxiolytic-like activity of rubimetide was successfully mediated by the PGD2-DP1 receptor, adenosine-A_{2A} receptor and $GABA_A$ receptor systems downstream of the FPR2 receptor.[88]

3.2. Peptides from soy proteins and ovolin

Apart from mediating the anxiolytic-like activity after interaction with the δ opioid receptor such as rubiscolin-6, there are certain peptides that have affinity towards the μ opioid receptor for this activity. For example, soymorphin-5 (YPFVV), -6 (YPFVVN) and -7 (YPFVVNA) derived from soy β-conglycinin are specific for the μ opioid receptor (IC_{50} 17, 39 and 47 μM, respectively). Soymorphin-5 and -6 exhibited an anxiolytic-like activity when administered in mice by i.p. (3–10 mg kg^{-1}) and p.o. (10 mg kg^{-1}; 16 μmol kg^{-1} and 13,5 μmol kg^{-1}, respectively) routes, as they significantly enhanced the percentage of the time spent in open arms in the EPM test. Soymorphin-7 displayed an anxiolytic activity only after a 30 mg kg^{-1} (37 μmol kg^{-1}) i.p. administration. Paradoxically, soymorphins given at higher doses did not cause any anxiolytic effect. In the case of soymorphin-7, higher dose requirement might be explained by its larger molecular size possibly decreasing the ability to penetrate the gut–blood barrier.[89] Human β-casomorphin-4 (YPFV) which also corresponds to soymorphin-4 and bovine β-casomorphin-5 in which the valyl carboxy-terminal residue of soymorphin-5 is replaced by a glutamyl residue showed no anxiolytic activity. Recently, it was shown that an undecapeptide FLSSTEAQQSY derived from soy β-conglycinin [βCGα(323–333)] displayed an anxiolytic-like activity in mice. The orally administered peptide (0.3 mg kg^{-1}; 0.24 μmol kg^{-1}) exhibited anxiolytic-like effects in the EPM test in sham-operated control mice but not in vagotomized mice. In addition, an increased c-Fos expression was also reported in the nucleus of the tractus solitarius (NTS), a visceral sensory nucleus in the brain that receives inputs from the vagus nerve, of the mice treated with βCGα(323–333) at a dose of 1.0 mg kg^{-1} p.o.[90] This study thus highlighted that some peptides can act at a central level *via* the gut–brain axis.

The tryptic digest of heat-treated ovalbumin, the major egg white protein, is also known to exhibit anxiolytic-like activity in the EPM test in mice. The percentage of time spent in and the number of visits to open arms were significantly increased in the EPM test after p.o. (47–160 mg kg^{-1}) and i.p. (16–47 mg kg^{-1}) administration of the ovalbumin tryptic digest. The anxiolytic-like activity of the tryptic digest was mainly due to the active fragment VYLPR, named ovolin that corresponds to the residues 280–284 of the hen ovalbumin. Ovolin is a pentapeptide that exhibited anxiolytic-like activity comparable to diazepam in the EPM test. The administration

of synthetic ovolin in mice by the oral (1 mg kg^{-1}; 1.55 μmol kg^{-1}), i.p. (0.3 mg kg^{-1}), or i.c.v. (0.3 nmol per mouse; about 0.2 μg per mouse) route also effectively improved the percentage of time spent in and the number of visits to the open arms in the EPM test. Moreover, in the open field test i.p. (0.1 mg kg^{-1}) injection of ovolin also increased the percentage of time spent in the center of circle while the locomotor activity remained unaffected like in the EPM test. In this case, the anxiolytic-like activity was inhibited by the cyclooxygenase inhibitor indomethacin (10 mg kg^{-1}), and by the antagonists BWA868C (60 μg kg^{-1}), SCH58261 (0.1 mg kg^{-1}), and bicuculline (5 mg kg^{-1}) of the receptors PGD_2-DP_1, adenosine A_{2A} and the GABA site of the $GABA_A$ receptor, respectively, usually involved in the emotional regulation. Although the affinities of ovolin (100 μM) for these receptors were negligible, it seemed that the anxiolytic-like activity of ovolin was mediated by PGD_2, produced as a result of increased cyclooxygenase (probably COX-2) activity, through its interaction with the DP_1 receptor coupled downstream to the adenosine A_{2A} and $GABA_A$ receptor systems.[91] It could be noted that the YLPR peptide displayed no activity, whereas the authors concluded in the publication dealing with the YL dipeptide that the lengthening of the YL chain on the carboxy-terminal part had no effect on the anxiolytic activity.[59]

3.3. Fish protein hydrolysate/Gabolysat®

Psychological (restraint type) and systemic (ether inhalation type) stressors have been applied in rats to test the anxiolytic-like activity of a commercial fish protein hydrolysate (Gabolysat PC60) obtained from the cod (*Gadus*) and mackerel (*Scomber*) fish. Exposure to these stressors resulted in the increased plasma levels of the adrenocorticotropic hormone, corticosterone (hormones of the pituitary-adrenal axis) as well as noradrenaline and adrenaline (catecholamine neurotransmitters). However, administration of Gabolysat PC60 (1200 mg per kg per day) for five consecutive days using an intragastric feeding tube drastically attenuated the increase in the plasma levels of adrenocorticotropic hormone similar to diazepam (valium, 1 mg kg^{-1}) after exposure to either ether inhalation (3 min) or restraint stress (30 min), whereas the corticosterone levels remained unaffected. Likewise, Gabolysat PC60 and diazepam administrations reduced the increase of adrenaline and noradrenaline levels in rats subjected to both types of stresses compared to the control group (carboxymethylcellulose 1%). Moreover, the GABA amount was slightly reduced in the hippocampus but significantly reduced in the hypothalamus of the Gabolysat PC60-treated rats after exposure to stress, indicating the increased release of GABA and its involvement in the anxiolytic-like activity.[92] Similarly, dogs fed on fish protein hydrolysate (GABOCEAN 3D PTP55) were assessed for fear and anxiety responses induced by noise using a thunderstorm model (locomotion measurement in the open field). It has been shown that the dogs receiving this hydrolysate displayed reduced fear and anxiety as manifested by decreased hyperactivity and a reduced cortisol response after exposure to stress.[93]

4. General discussion and conclusions

The bioactive peptides isolated from enzymatic hydrolysates of certain food proteins have numerous physiological effects on different biological functions such as antihypertensive, analgesic, memory consolidation, hypocholesterolemic, immunomodulatory, antithrombotic, antioxidant, or anxiolytic effects. These peptides might be involved in various physiological effects in humans long before the term “bioactivity” was defined. Some of these peptides are naturally formed in foods while others are formed in the digestive tract of the consumer. For example, α-CZP was detected 4 min after consumption of raw milk in the stomach of mini-pigs,[94] and the shorter peptide YLGYLEQ was found in the jejunum of healthy human volunteers after ingestion of casein.[95] Peptide genesis in the gastrointestinal tract may vary from person to person due to the matrix of administration and variability in protease relative concentrations.[96] Moreover, to exert their physiological effects *in vivo* after oral administration, the bioactive peptides may have to face various challenges. They may have to cross various physiological barriers to reach their target sites in an active form. This implies resistance to the gastrointestinal enzymes and brush border membrane peptidases as well as absorption through the intestinal epithelium with the possibility of hydrolysis by intracellular peptidases during the transcellular transport. Lastly, the peptides may have to confront the blood enzymes after their entry into the circulation.[97] The resistance of the bioactive peptides to these barriers is usually determined by sequential hydrolysis with pepsin and pancreatic enzymes under simulated gastrointestinal conditions and in *in vitro* studies with epithelial intestinal cells. Studies regarding the stability of food-derived anxiolytic peptides against these barriers are lacking. For instance, hydrolysis of the only studied peptide α–CZP (YLGYLEQLLR) by gastrointestinal enzymes under simulated digestion conditions revealed that a certain amount of the peptide was hydrolysed into shorter sequences. Among them, YLGYL and YLGYLEQ displayed a similar anxiolytic-like profile to that of the precursor peptide, but the mode of action was not strictly identical when modulation of the amygdala neuronal activity was compared between α-CZP and YLGYL.[32,57] These peptides were supposed to participate in the *in vivo* activity of α-CZP. This result indicates that the hydrolysis of a bioactive peptide in a shorter sequence does not necessarily lead to a loss of the biological activity. This hypothesis is further reinforced by the anxiolytic-like activities of the dipeptide YL and tripeptide YLG after oral intake in behavioral tests in mice.[33] Both the peptides correspond to the first 2 and 3 N-terminal residues, respectively, of α-CZP and its shorter fragments YLGYLEQ and YLGYL and thus show the importance of these residues in anxiolytic-like activity. However, the mode of action of α-CZP and its shorter fragments appears to be different from that of the di- and tripeptides. All these fragments have different physicochemical properties and some forms may resist proteolysis steps or cross physiological barriers more

easily. The physiological conditions also appear to be a main parameter in the action of bioactive peptides as the resistance towards hydrolysis by brush border peptidases and the transport across the Caco-2 monolayer cells of both peptides (α-CZP and YLGYLEQ) increased in the presence of bile salts. In contrast, a larger number and a greater variety of shorter peptides were formed in the absence of bile salts.[58] Generally, the transport of di- and tripeptides across the intestinal epithelium is mediated by the H^+-coupled peptide transporter PEPT1,[98] whereas larger peptides might get across by passive diffusion through the tight junctions (paracellular pathway)[99]or the transcellular route. It might be possible that α-CZP or its shorter fragments have traversed the intestinal epithelium by either paracellular or transcellular route to access $GABA_A$ receptors in the CNS. These peptides could also transmit signals to the brain *via* the afferent vagus nerve to initiate an anxiolytic-like activity, since it was shown that medium molecular weight peptides can also communicate *via* the gut–brain axis.[100] The anxiolytic-like activity after oral administration might also be explained by resistance to digestive proteases in the gastrointestinal tract, as in rubiscolin-6 (YPLDLF) and ovolin (VYLPR), where the presence of a Pro residue might offer a resistance to endogenous proteases. Another reason might be the transport of these peptides across the intestinal epithelial cells due to their low molecular weight (rubimetide MRW and β-LT HIRL). If the target is the CNS, should the amount of peptide that must reach this organ be large? Studies carried out with β-LT may provide a partial answer to this question. The anorexigenic activity of β-LT was observed in mice after administration of an oral dose of 500 mg kg^{-1} and an i.c.v. dose of 0.7 mg kg^{-1}, *i.e.* a ratio of approximately 700 between both routes (Table 3). This value should be obtained with care because many factors influence the absorption, distribution and bioavailability of a molecule in the body. Nonetheless, this suggests that the active oral doses of bioactive peptides are not dietary ones. One molecule out of 100 orally taken molecules might be enough to trigger the biological activity.

Consisting with these data, the peptides of food origin with anxiolytic activity could be used for the development of functional foods as well as nutraceuticals. Although further work is required to understand the minimum sequence required for bioactivity as well as to enhance our knowledge of the molecular mechanisms underlying the modulation of the CNS by these peptides, their importance as modulators of physiological responses cannot be ruled out.

Table 1. Pre-clinical studies in rodents showing the anxiolytic effects of α-casozepine and casein tryptic hydrolysate in different anxiety models

Protein hydrolysate /peptide	Description of study	Species	Dose	Results	Ref.
α_{s1}-CN hydrolysate	EPM	Wistar rats	3 mg kg^{-1}, i.p. 30 min before test	↑Percentage of entries into open armsa	30
	CDB	Wistar rats	3 mg kg^{-1}, i.p. 30 min before test	↓Probe burying durationa ↓Percentage approaches towards the probe followed by retreats ↓Number of heads stretching towards the probea	
	Evaluation of anticonvulsant activity	Wistar rats	3 mg kg^{-1}, i.p. 30 min before test	↓Seizure severityb ↑Seizure latencyb ↓Seizure durationb	
i-CTH (Lactium®)	CDB	Wistar rats	15 mg kg^{-1}, p.o. 60 min before test	↓Probe burying duration ↓Latency of the first probe approach after shockc Latency of the first probe contact (NS)c	31
	EPM	Wistar rats	15 mg kg^{-1}, p.o. 60 min before test	↑Percentage of the time spent in open armsc ↑Percentage of open arm entriesc (i) Total arm entries, (ii) total and open arm head dips, (iii) total and open arm rears and (iv) total and open arm rearing duration (NS)c	
i-CTH (Lactium®)	CDB	Wistar rats	15 mg kg^{-1}, p.o. 60 min before test	↓Probe burying duration ↓Number of retreats away from the probe ↓Percentage of approaches towards the probe followed by retreatsc ↑Duration of approaches towards the probec	35
	CDB for tolerance evaluation	Wistar rats	15 mg kg^{-1}, p.o. twice daily during 7 days and 1 h before test on day 8	↓Probe burying durationc Lack of induced tolerance	
	Passive avoidance test	Wistar rats	150 mg kg^{-1}, p.o. 60 min before test	↑Latencies before entering the dark compartment compared to diazepam Lack of anterograde amnesia	
	Conditioned place preference	Wistar rats	30 mg kg^{-1}, p.o. received on days 4, 6, 8, 10 and 12, 60 min before test	↓Time spent in the non-preferred compartment compared to diazepam Lack of addiction	
α-CZP	CDB	Wistar rats	0.4 mg kg^{-1}, i.p. 30 min before test	↓Probe burying duration and percentage approaches towards the probe followed by retreatsa ↓Number of heads stretching towards the probe (DNS)	30
α-CZP	LDB	Swiss mice	1.0 mg kg^{-1}, i.p. 30 min before test	↑Time spent in the lighted box ↑Number of rears in the lighted compartment	34
α-CZP	EPM	Wistar rats	1.0 mg kg^{-1}, i.p. 40 min before test	↑Percentage of open arm entries and the time spent in open armsa Total arm entries and the latency time before entering into the closed arm (NS)a	32
	LDB	Wistar rats	1.0 mg kg^{-1}, i.p. 30 min before test	↑Percentage of (i) entries and (ii) time spent in the lighted compartmenta ↑Number of rears in the lighted compartment	
	CDB	Wistar rats	0.7 mg kg^{-1}, i.p. 30 min before the test	↓Probe burying duration (NS)a	
YLGYLEQ α_{s1}-CN-(f91–97)	EPM	Wistar rats	0.7 mg kg^{-1}, i.p. 40 min before test	↑Percentage of (i) open arm entries and (ii) time spent in open armsa Total arm entries and latency time before entering into the closed arm (NS)a	32
	LDB	Wistar rats	0.7 mg kg^{-1}, i.p. 30 min before test	↑Percentage of (i) entries and (ii) time spent in the lighted compartmenta ↑Number of rears in the lighted compartmenta Total number of entries and latency time before entering into the lighted compartment (NS)a	
	CDB	Wistar rats	0.5 mg kg^{-1}, i.p. 30 min before test	↓Probe burying durationa	
YLGYL α_{s1}-CN-(f91–95)	LDB	Swiss mice	0.5 mg kg^{-1}, i.p. 30 min before test	↑Time spent in the lighted boxd ↑Number of rears in the lighted compartmentd	57
i-CTH (Lactium®)	Disturbance of sonorous (85 dB) and light–dark cycle (ten 5 min long episodes during 24 h) as well as cage tilt	Wistar rats	15 mg kg^{-1}, p.o. once a day for 8 days	↑Total sleep durationе Maintain slow wave sleep durationе ↑Paradoxical sleepe Corticosterone concentration (NS)e	52
i-CTH (Lactium®)	Pentobarbital-induced sleeping test	ICR mice	150 mg kg^{-1} p.o. 2h before test	↑Sleep duration induced by pentobarbital sodium	53
	Electrophysiological recording (EEG)	Sprague-Dawley rats	150 mg kg^{-1} p.o. 60 min before the test	↑Delta total power as compared to the control	
i-CTH (Lactium®)	Pentobarbital-induced sleeping test	C57BL/6 mice	240 mg kg^{-1} p.o. single dose, 30 min before the test	↑Sleep duration induced by pentobarbital sodium	37
	EEG recording	Sprague-Dawley rats	300 mg kg^{-1} p.o daily for 3 days	↓Sleep–wake cycle compared to the control ↑Total sleep ↑Theta power densities compared to the control	

i-CTH: industrial α_{s1}-CN tryptic hydrolysate; EPM: elevated plus maze test; CDB: conditioned defensive burying paradigm; LDB: light/dark box; NS: non-significant; DNS: data not shown.*a* With respect to control subjects treated with NaCl 9‰.*b* With respect to control subjects treated with dimethyl isosorbide ether.*c* With respect to control subjects treated with methylcellulose.*d* With respect to control subjects treated with the vehicle (0.2% methylcellulose and 1% glycerol).*e* With respect to control subjects treated with total bovine milk proteins.

Table 2. Pre-clinical studies in rodents showing the anxiolytic effects of α-casozepine and casein tryptic hydrolysate in different anxiety models

Protein hydrolysate/peptide	Design of study	Subjects	Dose	Results	Ref.
Industrial CTH (Zylkene)	Double-blind placebo-controlled trial	Cats	15 mg per kg per day p.o. for 56 days	↑Overall behavioral score[a] ↑Social behavior with familiar and non-familiar people[a] Fear, aggression and autonomic signs (NS)[a] but change in slope for fear and autonomic signs Owners' evaluation of success and failures (NS)[a]	38
CALM FELINE (dried hydrolysed casein and L-tryptophan supplemented diet)	Randomized double-blind placebo-controlled trial	Cats	15 mg per kg per day for 8 weeks	↓Urinary cortisol concentration[b] ↑Ratio of plasma tryptophan to large neutral amino acids[b] no change in plasma cortisol levels	39
Industrial CTH (Zylkene)	Double-blind randomized comparative trial	Dogs	15 mg per kg per day p.o. for 56 days	↓Average score for emotional disorders in dogs similar to selegiline hydrochloride Equally effective in treating anxiety disorders as selegiline hydrochloride as assessed by owners	40
Diet containing industrial CTH	Randomized double-blind placebo-controlled trial	Beagle dogs	"*Ad libitum*" daily intake for 60 days	↓Overall reactivity evaluation form score in anxious dogs[c] ↓Exploratory behavior and plasma cortisol level ↑Environment oriented[c]	41
CALM CANINE (industrial CTH and L-tryptophan supplemented diet)	Single-blind crossover trial	Dogs	20 mg per kg per day CTH for 15 kg dog, 16 weeks trial	↓Anxiety-related behaviors like stranger-directed aggression and fear, nonsocial fear and touch sensitivity[d] ↓Increased urine cortisol to creatinine ratio in post stressor dogs[d]	42
Industrial CTH (Zylkene)	Blind study Transition from semi-feral to domestic management and handling	Shetland-ponies	1000 mg per day p.o. during 2 weeks with 5 days pre-treatment	↑Compliant behavior and learning efficiency during acclimation and training in domestic stressful situations	45
Industrial CTH (Zylkene)	Blind study Measurement of compliance and comfort with routine health care procedures	Mares	2000 mg per day p.o. for 5 days	Improvement in compliance and/or comfort scores for aversions like examination room entry, eye medication, intranasal treatment, jugular stick, lip twitch application and trailer loading	46
Industrial CTH (Lactium®)	Randomized double-blind placebo-controlled trial	Healthy males	3 × 400 mg p.o. 12 h apart	↑SBP and DBP mean percentages in both Stroop Test and CPT[e] Stable heart rate ↓Plasma cortisol concentration[e]	47
Industrial CTH (Prodiet F200)	Randomly-assigned double-blind	Women	150 mg per day p.o. for 30 days	↑SBP compared to the control Stable heart rate	48
Industrial CTH (Lactium®)	Double-blind randomized crossover placebo-controlled trial	Women	150 mg per day p.o. for 30 days	↓Stress related symptoms for dimensions of (i) digestion, (ii) intellectual symptoms[e] (iii) cardiovascular (iv) emotional and (v) social problems[e]	49
Industrial CTH (Lactium®)	Double-blind placebo-controlled parallel study	Men and women	1 capsule per day (2.7 mg of α-CZP) for 28 days	↓Sleep quality score, sleep latency and day time dysfunction[f]	54
Target 1® (Dietary supplement contains CTH)	Double-blind randomized placebo-controlled trial	Males and females	2 tablets per day for 12 weeks	Improved score for burnout scales, *i.e.* Beck Depression Inventory, BMS-10, MBI-HSS fatigue, MBI-HSS depersonalization and task management[g]	51
Industrial CTH (Lactium®)	Double-blind randomized placebo-controlled crossover trial	Men and women	1 capsule per day (300 mg CTH) for 4 weeks, 4 weeks again to the counterpart after washout	↑Total sleep time and sleep efficiency ↓Sleep latency and wake up sleep onset	55
Supplement (CTH + L theanine)	Randomized double-blind placebo-controlled trial	Men and women	1 capsule per day (150 mg CTH) for 4 weeks	↓Sleep quality score, sleep latency and day time dysfunction[e] ↓Anxiety, stress and depression	56

CPT: cold pressor test; CC: cortisol concentration; SBP: systolic blood pressure; DBP: diastolic blood pressure; α-CZP: α-casozepine; BMS-10: Burnout Measure Short version; MBI-HSS: Maslach Burnout Inventory-Human Services Survey.*a* Compared to placebo.*b* Compared to the control diet.*c* Compared to placebo, *i.e.* (protein 25%, crude fat 10%, starch 41.6%, dietary fiber 9.9%, crude fiber 4.2%, moisture 8%).*d* Compared to the control diet (a mixture of dry matter, ash, nitrogen free extract, protein, fat, crude fiber, linoleic acid, arachidonic acid, isoleucine, leucine, phenyalanine, tyrosine, valine, and tryptophan).*e* Compared to placebo, *i.e.* skimmed milk powder.*f* Compared to placebo, *i.e.* (dextrin, gelatin, moisture, titanium dioxide, ash).*g* Compared to placebo.

Table 3. Activities of β-LT and physiological routes potentially implicated in its actions

Activity	Species	Mode of administration	Dose	Potential site of action	Ref.
Hypertensive	Rat	i.v.	30 mg kg^{-1}	NTS_2	70
Antinociceptive	Mice	i.c.v.	5.7 mg kg^{-1}	NTS_2 and D_1 downstream	101
			[a]		
		s.c.	300 mg kg^{-1}		
Hypocholesterolemic	Mice	i.p.	30 mg kg^{-1}	NTS_2 and D_2 downstream	102
		p.o.	100 mg kg^{-1}		
Stress reduction after conditioning	Mice	i.p.	30 mg kg^{-1}	NTS_2 and may be HPA axis	76
Memory consolidation	Mice	i.c.v.	1.6 mg kg^{-1}	D_2 without direct interaction	77
			[b]		
		p.o.	500 mg kg^{-1}		
Anorexigenic	Mice	i.c.v.	0.7 mg kg^{-1}	CRF and CGRP downstream without direct interaction	103
			[c]		
		i.p.	100 mg kg^{-1}		
		p.o.	500 mg kg^{-1}		
Anxiolytic	Mice ddY	i.p.	10 mg kg^{-1}	NTS_2 and D_1 downstream	75
		p.o.	3–10 mg kg^{-1}		

a 200 nmol injected for an average weight of 19 g. *b* 60 nmol injected for an average estimated weight of 20 g (4 week old male mice) *c* 40 nmol injected for an average estimated weight of 30 g (7 week old male mice); the molecular mass of β-LT is 537.66 g mol^{-1}. i.c.v.: intracerebroventricular; i.p.: intraperitoneal; i.v.: intravenous; p.o.: *per os*; s.c.: subcutaneous; HPA: hypothalamic-pituitary-adrenal axis; CGRP: calcitonin gene-related peptide; CRF: corticotrophin releasing factor; D_1: dopamine D_1 receptor; D_2: dopamine D_2 receptor; NTS_2: neurotensin receptor of low affinity.

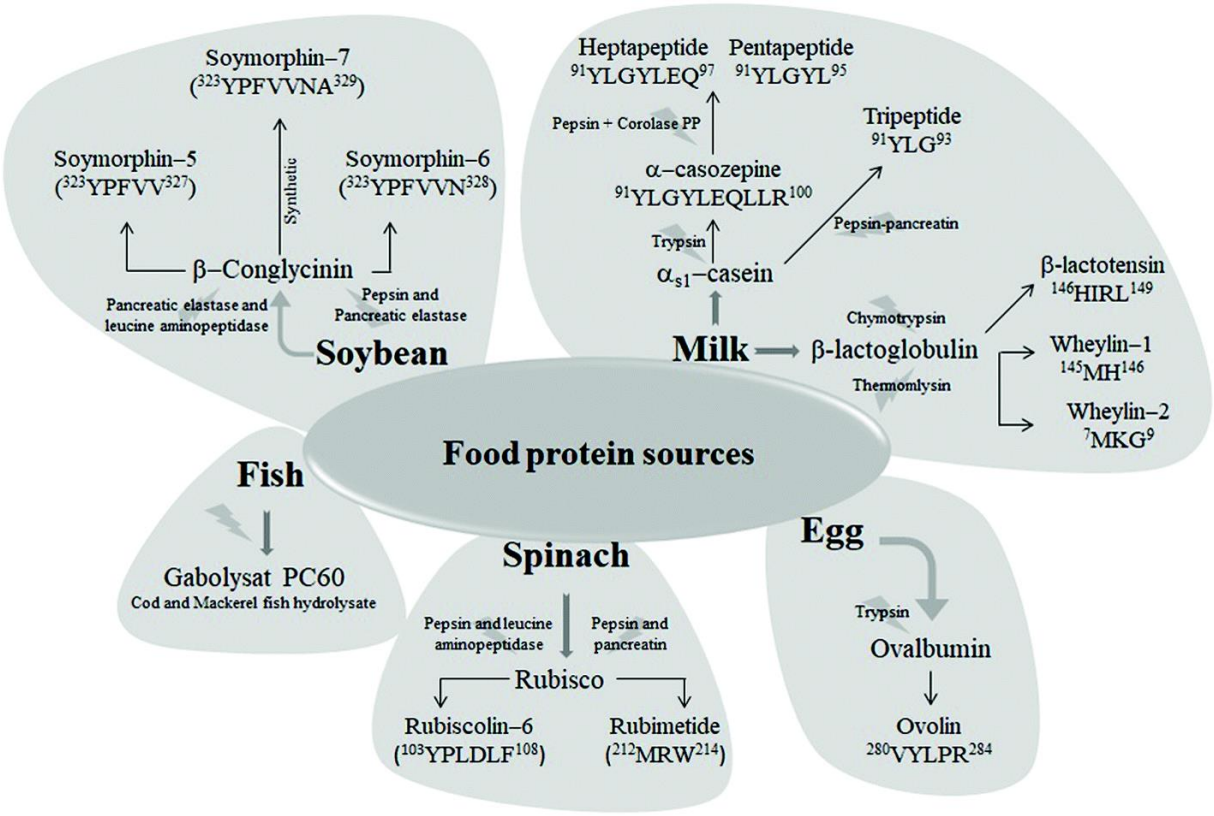

Figure 1. Hydrolysates/peptides from food proteins with anxiolytic-like activity

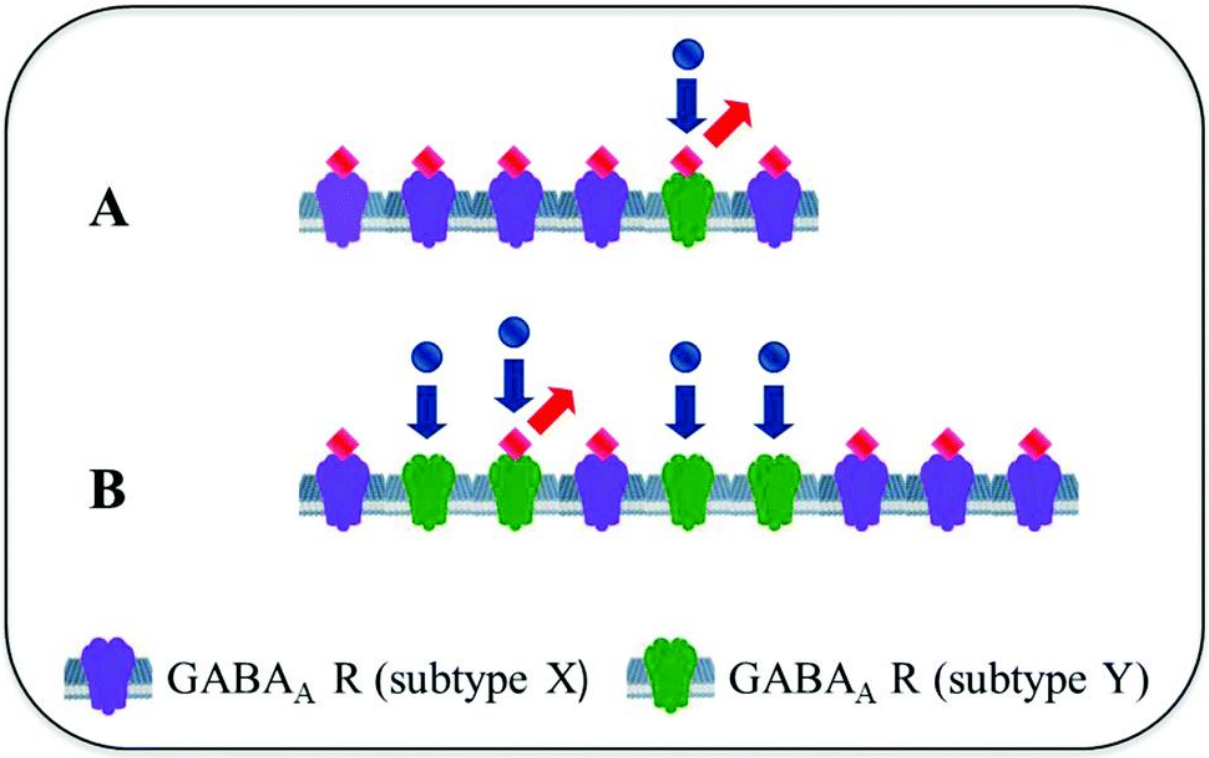

Figure 2. Schematic representation of the possible hypotheses to explain the phamacological action of α-CZP

Indication: Numbers correspond to the PCB congeners. Framed congeners are dioxin-like PCBs. Bold numbers were congeners transferred at a high level ranking from 38 to 78% and from 30 to 80% respectively for milk and eggs.

(●) for the $GABA_A$ receptor ($GABA_A$ R) despite lower affinity than diazepam. (A) $GABA_A$ R subtype Y of major pharmacological action but present in a low quantity is targeted by α-CZP. (B) $GABA_A$ R subtype Y present in significant quantity is targeted by α-CZP but possesses low affinity for flunitrazepam (◆)

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7. Conflicts of interest

There are no conflicts of interest.