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IMPACT OF THE ADMINISTRATION OF α-CASOZEPINE, A BENZODIAZEPINE-LIKE PEPTIDE FROM BOVINE α_{S1}-CASEIN, AND OF A PROTEOLYSIS FRAGMENT ON NEURAL ACTIVITY IN MICE

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René Barjavel, L'Enchanteur

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ABSTRACT

α-casozepine (α-CZP) is a decapeptide that mediates the anxiolytic-like properties of the tryptic hydrolysate of bovine α_{s1}-casein. Different properties of α-CZP leads to consider this peptide close to the benzodiazepine family, the most commonly used anxiolytic molecules. In contrast, other results suggest a distinct mode of action between α-CZP and benzodiazepines, especially the fact that the peptide does not have side effects. Although a central action remains the main hypothesis of the mode of action of α-CZP, no regulation of the brain activity has been shown before. The work achieved in this thesis displayed the fact that the anxiolytic-like properties of α-CZP, after a single intraperitoneal injection of the peptide, are associated with a modulation of cerebral activity in several regions linked to anxiety regulation in mice brains, such as the amygdala, the hippocampal formation, the accumbens nucleus and some nuclei of the hypothalamus or raphe. Besides, these modulations of neural activity are not exactly the same as those obtained after an injection of diazepam, a reference benzodiazepine, or YLGYL, a derivative of α-CZP, even though observed behaviours are similar. Eventually, it has been demonstrated that an anxiety-inducing situation is needed to trigger the central effects of α-CZP. This work allowed a better understanding of the mode of action of a bioactive peptide from alimentary origin that has a positive action on its consumer's mood and behaviour.

Keywords: *α-casozepine, bioactive peptide, anxiety, neural activity, animal behaviour*

RÉSUMÉ

L'α-casozépine (α-CZP) est un décapeptide porteur des propriétés anxiolytiques de l'hydrolysat trypsique de caséine α_{s1} bovine. Différentes propriétés ont pu rapprocher ce peptide de la famille des benzodiazépines, les anxiolytiques les plus prescrits. Cependant, certaines différences, dont notamment une absence d'effets secondaires, permettent aussi de distinguer l'α-CZP des benzodiazépines. Bien que de nombreux éléments laissent penser qu'une action centrale reste l'hypothèse principale du mécanisme d'action de l'α-CZP, aucune régulation de l'activité de zones cérébrales n'avait été montrée jusqu'à présent. Ce travail de thèse aura donc pu montrer que les propriétés anxiolytiques de l'α-CZP sont associées à une modification de l'activation neuronale chez la souris, après une unique injection intrapéritonéale, dans différentes régions impliquées dans la régulation de l'anxiété, notamment l'amygdale, la formation hippocampale, le noyau accumbens et certains noyaux de l'hypothalamus et du raphé. De plus, ces modifications de l'activation neuronale ne sont pas exactement les mêmes que celles observées avec le diazépam, une benzodiazépine de référence, ni de celles obtenues avec YLGYL, un peptide dérivé de l'α-CZP, bien qu'il existe des similitudes dans le comportement de l'animal suite aux différents traitements effectués. Enfin, il a été démontré qu'une situation anxiogène est indispensable pour révéler cet effet central. L'ensemble de ce travail aura permis d'avancer dans la compréhension du mode d'action d'un peptide alimentaire ayant des effets positifs sur le comportement et les émotions de son consommateur.

Mots-clefs : *α-casozépine, peptide bioactif, anxiété, activité cérébrale, comportement animal*

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LIST OF ABBREVIATIONS

5-HT$_{1A}$	Serotonin type 1A receptor
Acb	Accumbens nucleus
ACTH	Adrenocorticotropin hormone
AD	Anxiety disorder
AFSSA	Agence Française de Sécurité Sanitaire des Aliments
ANSM	Agence Nationale de Sécurité du Médicament et des produits de santé
AVP	Arginine-vasopressin hormone
BIS	Behavioural inhibition system
BLA	Basolateral nucleus of the amygdala
BMA	Basomedial nucleus of the amygdala
BNST	Bed nucleus of the stria terminalis
BOLD signal	Blood-oxygen-level dependant signal
BZD	Benzodiazepine
CDB	Conditioned defensive burying
CeA	Central nucleus of the amygdala
CH	Tryptic hydrolysate of bovine α_{s1}-casein
CNS	Central nervous system
CRH	Corticotropin releasing hormone
D_1	Dopamine type 1 receptor
Da	Dalton
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, fifth edition
EEG	Electroencephalography
FDA	American Food and Drug Administration
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
$GABA_A$	GABA type A receptor
$GABA_B$	GABA type B receptor
$GABA_C$	GABA type C receptor
GABA-T	GABA transaminase enzyme
GAD	Glutamic acid decarboxylase enzyme
GAD	Generalised anxiety disorders

GRAS	Generally Recognised as Safe
HPA axis	Hypothalamus-pituitary-adrenal axis
i.p.	Intraperitoneal
IC_{50}	Half maximal inhibitory concentration
iCH	Industrial tryptic hydrolysate of bovine αs1-casein (Lactium®)
MeA	Medial nucleus of the amygdala
MEG	Magnetoencephalography
NMR	Nuclear magnetic resonance
OCDs	Obsessive-compulsive disorders
PAG	Periaqueductal grey
PET scan	Positron emission tomographjy scan
PFC	Prefrontal cortex
PVN	Paraventricularnucleus of the hypothalamus
SDS	Sodium dodecyl sulfate
USDA	U.S. Department of Agriculture
α-CZP	α-casozepine
α_{s1}*-CN*	α_{s1}-casein
β-CM	β-casomorphin
κ-CN	κ-casein

FOREWORD – FOOD FOR MOOD

Despite food is traditionally being considered as a way to provide energy and nutrients to the organism, its ability to avert or protect against diseases is also starting to be acknowledged (Gómez-Pinilla, 2008). Amongst the different potential health effects of food, one may be its action on the central nervous system, and more specifically, its psychological action. Over the past decades, a clearer link has been made between food and mood, mood being a long lasting emotional state. Moods are commonly described as having either a positive ('being in a good mood') or a negative valence ('being in a bad mood'). Negative moods are often associated to depression, anxiety, aggression, and/or poor self-esteem for instance, having an impact on everyday lifestyle (Howren and Suls, 2011).

There's a growing body of evidence (as well as personal experiences) pointing out the fact that mood directly influences what we eat (for instance, with consumption of 'comfort foods' while being under a negative mood, despite this being debated (Wagner *et al*, 2014)). However, the converse may also be true, indicating that several foods could also have an effect, positive or negative, on mood. The underlying principle would be that substances in food interact with organism's chemistry to trigger mood changes. This effect can directly be due to some specific pharmacological components on the food itself, such as theanine or caffeine (Einöther and Martens, 2013; Nehlig, 2016; Wang *et al*, 2015). However, the composition in macro- and micronutrients may also exerts effects on mood.

As an example, the effects of omega-3 fatty acids on psychological disorders, and especially depression, have been extensively described (Deacon *et al*, 2017). Decreased plasma omega-3 fatty acids was observed amongst patients with major depression, while a supplementation with this same polyunsaturated acids would help to reduce depressive symptoms (Casper, 2004). The American Psychiatric Association's Committee on Research on Psychiatric Treatments gave some guidelines for the use of supplementation in omega-3 fatty acids to prevent and treat mood disorders (Freeman *et al*, 2006). Tryptophan, an essential amino acid, has also been linked to mood regulation as a depletion in the diet triggered lowering of mood, irritability, tiredness and distractibility even within healthy subjects (Casper, 2004). Eventually, Wurtman and Wurtman suggested that carbohydrates might help to relieve depression as a high carbohydrates diet is thought to favour the absorption of tryptophan in the brain (Benton and Donohoe, 1999; Ottley, 2016).

Nutrition has then been enriched with the concept of 'functional food', where food is supposed to have a positive effect on the consumer's health (Roberfroid, 1999). The fact that the act of feeding is a human routine emphasizes the power of dietary factors to modulate mood and mental health. This generates the possibility that adapted dietary habits may be a strategy to enhance mood or prevent psychological disorders. However, this must not fall into the trap of over-interpreting Hippocrates' famous citation: 'let food be thy medicine and medicine be thy food', and thinking that food will one day substitute every drug in the market, as the complexity of the phenomenon is yet to be fully understood and that particular food won't have a dramatic impact on moods and behaviours.

Amongst the different components present in food that may have a positive effect on health are bioactive peptides. Peptides offer new therapeutic opportunities, having specific combining actions compared to small molecules. Some have been shown to act on the central nervous system, both directly or indirectly. More specifically, a bioactive peptide coming from bovine milk casein has been identified to have anxiolytic-like properties: α-casozepine (Miclo *et al*, 2001). Although the different results obtained support the hypothesis of a central target, the mode of action of the peptide has not been elucidated before

This thesis then will try to decipher the central mode of action of α-casozepine. Thus, before getting into scientific results, the general introduction is going to untangle the different level of organisation that will allow a better understanding of the results, and how they find their place in a bigger picture. The first section will then introduce the therapeutic opportunities of bioactive peptides, and will focus specifically on the central effects of peptides coming from milk proteins. The second section, will next focus on a specific neurotransmitter, GABA, and its specific role in anxiety regulation, before introducing this thesis central piece of interest: α-casozepine. To dig deeper into anxiety, the third section will define this phenomenon, its associated psychological disorders, and have a glance at its central regulation. Eventually, linking up the scientific rationale and the work undertook during this thesis, the fourth and last section will concentrate on specific animal models used to either study anxiety, or neural activity.

PART I – GENERAL INTRODUCTION

1 BIOACTIVE PEPTIDES FROM MILK PROTEINS

Contents

Some evidences stated that food cannot be only limited to the mere sum of both macronutrients and micronutrients, and that many other biological effects can be provided *via* it. Some molecules have already been identified as having an impact on human health (Nongonierma and FitzGerald, 2015). A number of these compounds are **bioactive peptides** coming from hydrolysis of food proteins.

Bioactive substances are 'food components that can affect biological processes or substrates and hence, have an impact on body function or condition and ultimately health' (Möller *et al*, 2008; Schrezenmeir *et al*, 2000). This definition is usually developed with two precisions: (1) the impact should affect a measurable biological effect at physiologically realistic level; (2) the bioactivity being measured must affect health in a beneficial way, excluding then potentially damaging effects (toxicity, allergy or mutagenicity). Bioactive peptides have been defined as 'specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health' (Kitts and Weiler, 2003; Korhonen, 2009).

This section will then review some therapeutic effects of milk bioactive peptides, concentrating on peptides which have a central action.

1.1 Advances in therapeutic molecules: peptide-based drugs

As it is going to be broached later on (cf. ***2.2.1*** and ***3.3.2***), most of the drugs developed since nowadays were small molecules, the 20th century being often called the 'chemistry era'. Drugs were supposed to be only small molecules due to Lipinski's rule-of-five (Lipinski, 2004a, 2004b; Lipinski *et al*, 2001): no more than 5 H-bond donors, no more than 10 H-bond acceptors, molecular weight under 500 Da, and calculated logP[1] no greater than 5. This rule stated the properties that would make a molecule a likely orally active drug. Peptides were then far to be considered as potential efficient drugs.

In the later part of the 20th century, a new class of therapeutics emerged, with fewer side effects. This new class is referred as 'biologics', including protein-based molecules such as

[1] log(concentration in octanol/concentration in water); act as an indicator of the potency of a molecule to accumulate in biological membranes; the higher the ratio, the higher the bioaccumulation risk is

insulin, antibodies or growth factors. However, they are not suitable for oral administration as they disobey the rule-of-five.

Small molecules (<500 Da) and biologics (>5000 Da) are then separated by a significant gap in molecular weight (***FIGURE 1.1***). Molecules filling this gap have not been used for the development of new therapeutic drugs since recently, and peptides fall into this category.

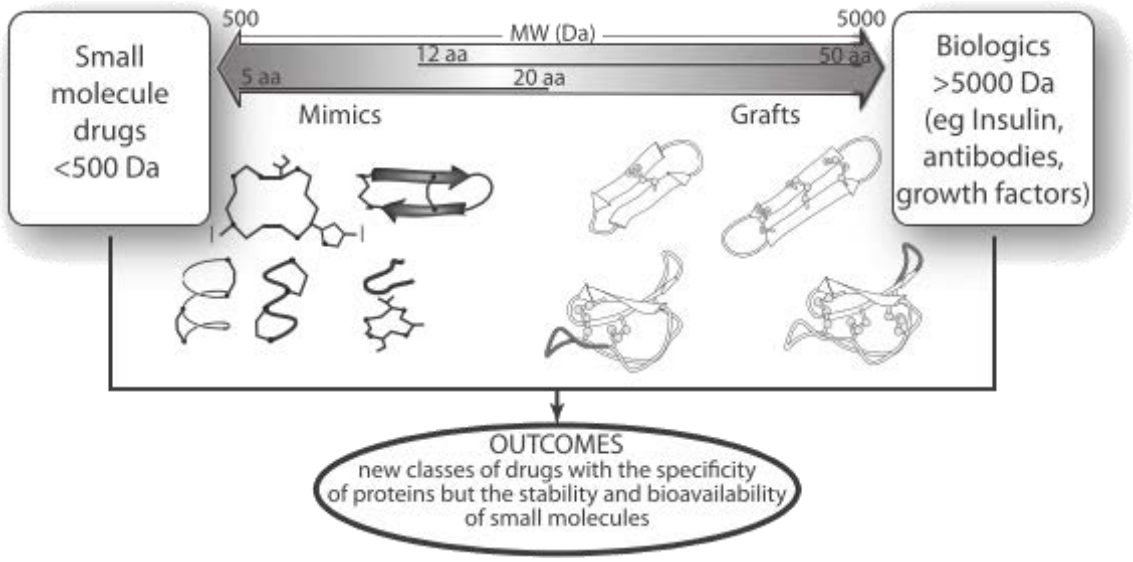


FIGURE 1.1 – Schematic illustration of the molecular weight (MW) gap between conventional small molecule drugs (<500 Da) and biologics (>5000 Da).
(Craik *et al*, 2013).

Peptides share advantages of both drug classes: they have the same specificity and potency of biologics associated with a smaller size and cheaper cost as small molecules. ***TABLE 1.1*** sums up the strengths and weaknesses of peptides used as drugs. A majority of these peptides are smaller than 20 amino acid residues in size (Vlieghe *et al*, 2010).

Despite a large amount of these peptides being synthetic peptides (Vlieghe *et al*, 2010) or derived from natural defences of organisms such as toxins (Vetter *et al*, 2011), an increasing number of them is coming from food (Yoshikawa, 2015), which might settle the problem of oral administration.

TABLE 1.1 – Analysis of the strengths, weaknesses, opportunities, and threats of naturally occurring peptides in their use as therapeutics.
(Craik *et al*, 2013; Fosgerau and Hoffmann, 2015).

Strengths	Weaknesses
- Good efficacy, safety and tolerability	- Chemically and physically instable
- High selectivity and potency	- Prone to hydrolysis and oxidation
- Predictable metabolism	- Tendency for aggregation
- Shorter time to market	- Short half-life and fast elimination
- Lower attrition rates	- Usually not orally available
- Standard synthetic protocols	- Low membrane permeability

1.2 Bioactive peptides derived from bovine milk proteins

Milk is an essential vector for the delivery of nutrients to the newborn but many beneficial effects that go beyond this role were also attributed to it: 'bioactive substances in milk and colostrum is mother language on a substrate basis' (Schrezenmeir *et al*, 2000). As such, several bioactive peptides are derived from bovine milk proteins.

Bioactive peptides derived from milk can be released *via* different ways: *in vitro* enzymatic hydrolysis, microbial fermentation during food process and/or *in vivo* gastrointestinal digestion (Bhat *et al*, 2015; Korhonen, 2009, 2013; Mohanty *et al*, 2015). The use of different enzymes may lead to different released peptides as the cleavage sites differ between microbial enzymes or digestive enzymes (***FIGURE 1.2***) (Möller *et al*, 2008).

Numerous different activities have been highlighted for milk bioactive peptides (***FIGURE 1.3***): cardiovascular health, digestive health, immune defence, impact on the nervous system, bone health, dental health, or weight management (Bhat *et al*, 2015; Hernández-Ledesma *et al*, 2013; Mohanty *et al*, 2015; Möller *et al*, 2008; Nagpal *et al*, 2011; Nongonierma and FitzGerald, 2015). A number of increasing clinical studies have assessed the effects of these peptides in human health (Bouglé and Bouhallab, 2015). A few of them are already being sold as dairy products or ingredients with health claims (***TABLE 1.2***) (Korhonen, 2009).

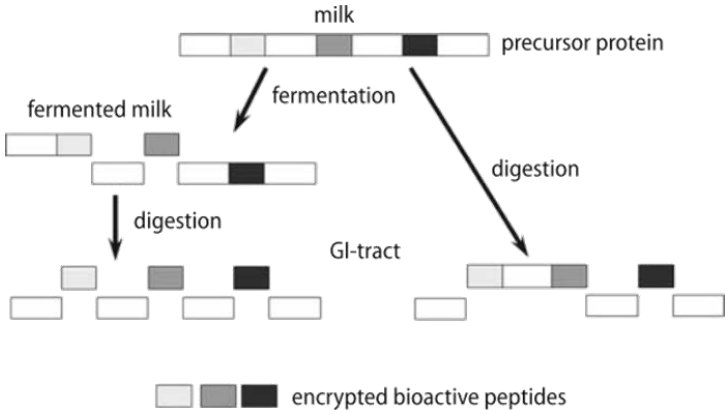


FIGURE 1.2 – Scheme of possible differences between peptides released from precursor proteins by fermentation and/or gastrointestinal digestion.

Adapted from (Möller *et al*, 2008).

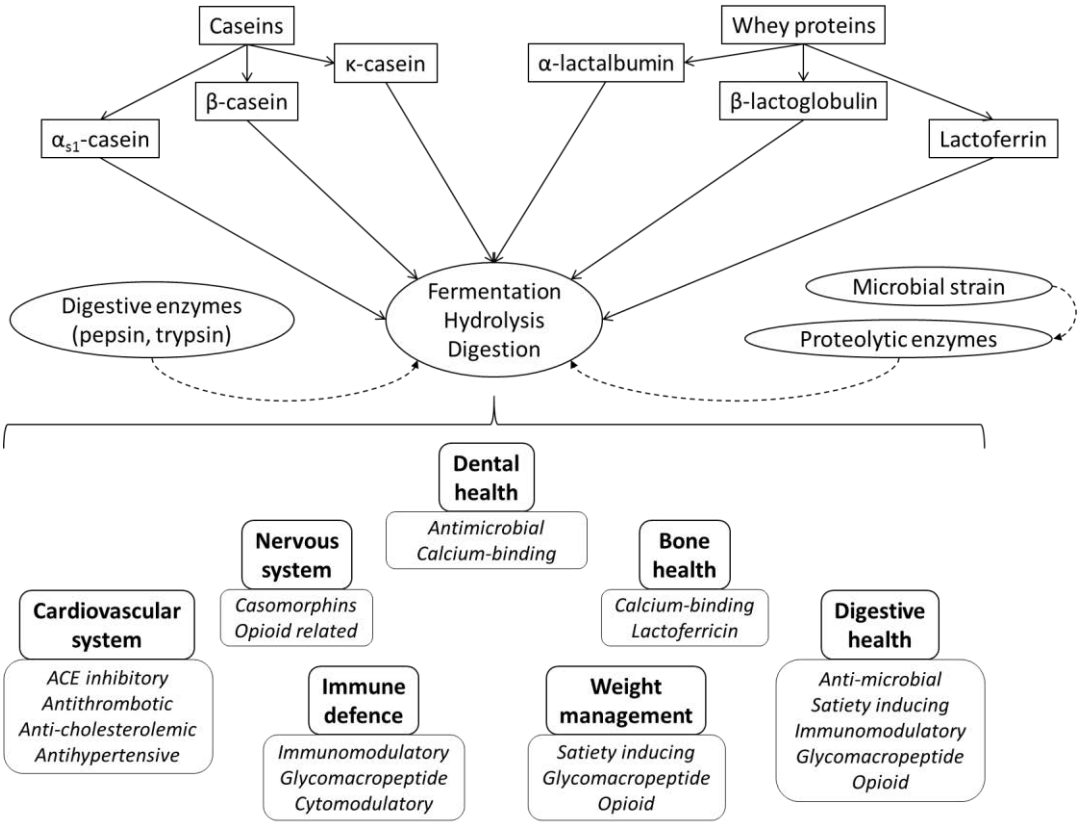


FIGURE 1.3 – Formation of bioactive peptides from major milk proteins and their potential health targets.

Adapted from (Korhonen, 2009; Park and Nam, 2015).

TABLE 1.2 – Commercial dairy products and ingredients with health or function claims based on bioactive peptides.

Adapted from (Korhonen, 2009; Korhonen and Pihlanto, 2006).

Brand name	Type of product	Claimed functional bioactive peptide	Health/claim claims	Manufacturer
Calpis	Sour milk	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ-casein	Reduction of blood pressure	Calpis Co., Japan
Evolus	Calcium enriched fermented milk drink	Val-Pro-Pro, Ile-Pro-Pro, derived from β-casein and κ-casein	Reduction of blood pressure	Valio Oy, Finland
BioZate	Hydrolysed whey protein isolate	β-Lactoglobulin fragments	Reduction of blood pressure	Davisco, USA
BioPURE-GMP	Whey protein isolate	κ-Casein f(106–169) (Glycomacropeptide)	Prevention of dental caries, influence the clotting of blood, protection against viruses and bacteria	Davisco, USA
PRODIET F200/Lactium	Flavoured milk drink, confectionery, capsules	α_{s1}-Casein f(91–100) (Tyr-Leu-Gly-Tyr-Leu- Glu-Gln-Leu- Leu-Arg)	Reduction of stress effects	Ingredia, France
Festivo	Fermented low-fat hard cheese	α_{s1}-casein f(1–6), (1–7), (1–9)	No health claim	MTT Agrifood Research Finland
Cysteine Peptide	Ingredient/hydrolysate	Milk protein-derived peptide	Aids to raise energy level and sleep	DMV International, the Netherlands
C12	Ingredient/hydrolysate	Casein derived peptide	Reduction of blood pressure	DMV International, the Netherlands
Capolac	Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
PeptoPro	Ingredient/hydrolysate	Casein derived peptide	Improves athletic performance and muscle recovery	DSM Food Specialties, the Netherlands
Vivinal Alpha	Ingredient/hydrolysate	Whey derived peptide	Aids relaxation and sleep	Borculo Domo Ingredients (BDI), the Netherlands
Recaldent	Chewing gum	Calcium casein peptone-calcium phosphate	Anticariogenic	Cadbury Adams, USA

Amongst the different milk proteins, caseins are the most abundant ones in ruminant milks and α_{s1}-casein is the rifest within them in bovine milk (***TABLE 1.3***). The sequence of B variant (UniProtKB – P02662) and its predicted secondary structure have been studied (***FIGURE 1.4***) and numerous peptides with different biologic actions can be obtained from it after a tryptic hydrolysis (***TABLE 1.4***).

TABLE 1.3 – Characteristics of bovine milk proteins.

	Number of residues	Concentration (g/L)
α_{s1}-casein	199	10
α_{s2}-casein	207	2.6
β-casein	209	9.3
κ-casein	169	3.3
β–lactoglobulin	162	2 to 4
α-lactalbumin	123	1.5
Lactoferrin	700	1 to 7

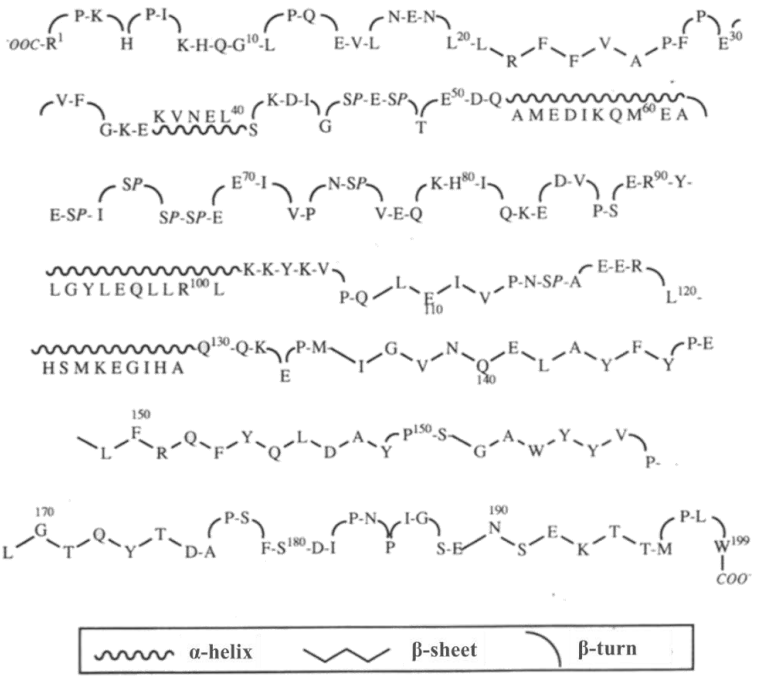


FIGURE 1.4 – Sequence (UniProtKB – P02662) and predicted secondary structure of bovine α_{s1}-casein B variant.

TABLE 1.4 – Bioactive peptides derived from α_{s1}-casein.

*can be obtained via a tryptic hydrolysis; S: indicates a phosphoseryl residue. Adapted from (Fitzgerald and Murray, 2006; Hata *et al*, 1998; Hernández-Ledesma *et al*, 2004; Meisel and Bockelmann, 1999; Miclo *et al*, 2001; Silva and Malcata, 2005).

Biological activity	Fragments	Sequences
Antihypertensive	f1-9	RPKHPIKHQ
	f23-24	FF
	f23-27	FFVAP
	f23-34*	FFVAPFPEVFGK
	f25-27	VAP
	f28-34	FPEVFGK
	f142-147	LAYFYP
	f146-147	YP
	f157-164	DAYPSGAW
	f194-199*	TTMPLW
Antimicrobial	f1-23	RPKHPIKHQGLPQEVLNENLLRF
Anxiolytic	f91-95	YLGYL
	f91-97	YLGYLEQ
	f91-100*	YLGYLEQLLR
Opioid	f90-95	RYLGYL
	f90-96	RYLGYLE
Immunomodulation	f1-23	RPKHPIKHQGLPQEVLNENLLRF
	f59-79*	QMEAESISSSEEIVPNSVEQK
	f194-199	TTMPLW
Mineral chelation	f43-58*	DIGSESTEDQAMEDIK
	f45-55	GSESTEDQAME
	f59-79*	QMEAESISSSEEIVPNSVEQK
	f66-74	SSSEEIVPN
	f106-119	VPQLEIVPNSAEER

We will now focus on bioactive peptides derived from bovine caseins, which have a positive effect on the nervous system.

1.3 Central effects of bioactive peptides derived from bovine caseins

A positive impact of milk consumption on mood and behaviour has been stated over the years. Indeed, it was identified that the consumption of a hot milk-cereal drink (Horlicks) lead to decreased movements during sleep (Laird and Drexel, 1934; Southwell *et al*, 1972). To be more specific, the same consumption of Horlicks led to a decrease of restlessness during sleep amongst young adults while it increased sleep total duration and decreased the periods of wakefulness amongst older adults (Brezinová and Oswald, 1972). Furthermore, studies concentrating on milk collected at night, have specified that it promotes sleep, due to a higher concentration in melatonin and tryptophan (Milagres *et al*, 2014; dela Peña *et al*, 2015; Valtonen *et al*, 2005). This comes back to the idea that some specific molecules in milk may specifically affect brain and its functioning.

Opioid peptides derived from β-casein were the first to be identified and were called β-casomorphins (Brantl *et al*, 1979, 1981; Henschen *et al*, 1979; Lottspeich *et al*, 1980). Different peptides were thus identified, released after hydrolysis of the peptide bond Val^{59}-Tyr^{60} of β-casein: ^{60}YPFP^{63}, ^{60}YPFPG^{64} (β-casomorphin-5, β-CM5), ^{60}YPFPGP^{65}, ^{60}YPFPGPL^{66} (β-casomorphin-7, β-CM7) and so on… Interestingly, compared to enkephalins (peptides that have both an affinity for opiate receptors and opiate-like effects which are inhibited by naloxone), they do not have the typical N-terminal sequence, YGGF (Quirion and Weiss, 1983). β-CM7 and β-CM5 have both an affinity for opiate μ-receptors (Chang *et al*, 1985), β-CM5 having an affinity 22 times lower than that of morphine while the affinity β-CM7 is 250 times lower than that of morphine (Brantl *et al*, 1981; Chang *et al*, 1982; Matthies *et al*, 1984). β-casomorphin-11 has been identified in porcine intestinal digesta (Meisel and Frister, 1989) while β-CM7 was identified in human small intestine (Svedberg *et al*, 1985). In β-casein A2 variant, the peptide bond between residue of isoleucine 66 and residue of proline 67 is more resistant towards enzymatic hydrolysis than that where residue of proline is substituted by a residue of histidine as in A1 or B variants. Thus, β-CM7 is more easily generated from β-casein A1 and B variants by gastrointestinal enzymes (Nguyen *et al*, 2015). After an intracarotid injection of [^{3}H]β-CM5 in rats, the peptide was accumulated in blood-brain barrier-free brain areas (Ermisch *et al*, 1983) while intravenous injection of β-CM7 has been shown to trigger

c-Fos, a neuronal activity marker (cf. ***4.2***), expression modulation in brain regions in schizophrenia and autism (Sun *et al*, 1999). Despite some increasing biological adverse effects (e.g. autism, cardiovascular diseases and type I diabetes) were found in both *in vitro* and *in vivo* studies for β-CM7, EFSA has stated that no causal effect could be made within humans (Noni *et al*, 2009).

Some other opioid peptides were isolated from pepsin hydrolysates of α_{s1}-casein sequence (Zioudrou *et al*, 1979). Identification of the peptides led to the following sequences: RYLGYL and RYLGYLE, respectively fragments α_{s1}-CN-(f90-95) and α_{s1}-CN-(f90-96) of α_{s1}-casein (Loukas *et al*, 1983). RYLGYL is more potent than its analogues. YLGYL and YLGYLE also displayed an affinity for opiate receptors, but they are relatively inactive, the arginine in the N-terminal end increasing the potency by a 5 to 35 factor. These peptides show little structural similarities with the previously described β-casomorphins. However, a common structural pattern has been identified as crucial for the affinity to μ receptors: a tyrosine in the N-terminal end with another aromatic amino acid (phenylalanine or tyrosine) in the 3rd or 4th position (Chang *et al*, 1981).

Eventually, three opioid antagonist peptides were identified in a tryptic hydrolysis of κ-casein (Chiba *et al*, 1989). The following sequences were hence identified: YIPIQYVLSR κ-CN-(f25-34), YPSYGLN κ-CN-(f35-41), and YPYY κ-CN-(f57-60).

Despite a great number of opiate peptides encrypted in the milk caseins sequences, the soothing effect of milk may also be explicated by other neurotransmitter systems. Indeed, the GABAergic system has been extensively described for its inhibitory effects in the central nervous system, and specifically for its role in anxiety regulation (Millan, 2003).

2 GABAERGIC SYSTEM AND α-CASOZEPINE

Contents

This section mostly focuses on one neurotransmitter system, the GABAergic system (cf. ***2.1***), its implication in anxiety regulation (cf. ***2.1.3***), and the associated pharmacological lever to deal with anxiety disorders, the benzodiazepines (cf. ***2.2***). The light will then be shed on a tryptic hydrolysate of bovine α_{s1}-casein, which exerts anxiolytic-like properties such as benzodiazepines (cf. ***2.3***) and eventually on α-casozepine, a decapeptide contained in this hydrolysate and sought to be the carrier of the anxiolytic-like properties (cf. ***2.4***).

2.1 GABA and $GABA_A$ receptors

2.1.1 Gamma-aminobutyric acid (GABA)

γ-aminobutyric acid (GABA), discovered in 1950 (Awapara *et al*, 1950), is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Siegel *et al*, 2006). GABA meet the five criteria for assignment as a neurotransmitter: existence in nerve terminals; release from electrically stimulated neurons; existence of mechanism to terminate the release of the neurotransmitter; application to neurons mimics the action of inhibitory nerves; existence of specific receptors. Between 30 to 40% of CNS synapses are GABAergic synapses (Bloom and Iversen, 1971; Guidotti *et al*, 1983). GABA is synthesised from glutamate by glutamic acid decarboxylase enzyme (GAD). It is catabolised with α-ketoglutarate by the GABA transaminase enzyme (GABA-T) to form succinic semialdehyde and glutamate (***FIGURE 2.1***) and succinic semialdehydde oxidised to succinic acid that enters into Krebs cycle.

A main part of GABA neurons are local interneurons. These local interneurons are specific neurons with small axons which form connections with nearby neurons to regulate small pieces of information, a great number of them being inhibitory GABAergic neurons (Whittington *et al*, 2000). GABAergic interneurons have been identified in rats in a specific brain region linked to anxiety regulation: the amygdala (and more specifically the central nucleus of the amygdala, cf. ***3.2.1.1***) (Sun and Cassell, 1993). These neurons may be implicated in the inhibition of central amygdala neurons projections to brainstem centres, leading to a more accurate regulation of the response initiated by the amygdala (Lee *et al*, 2013).

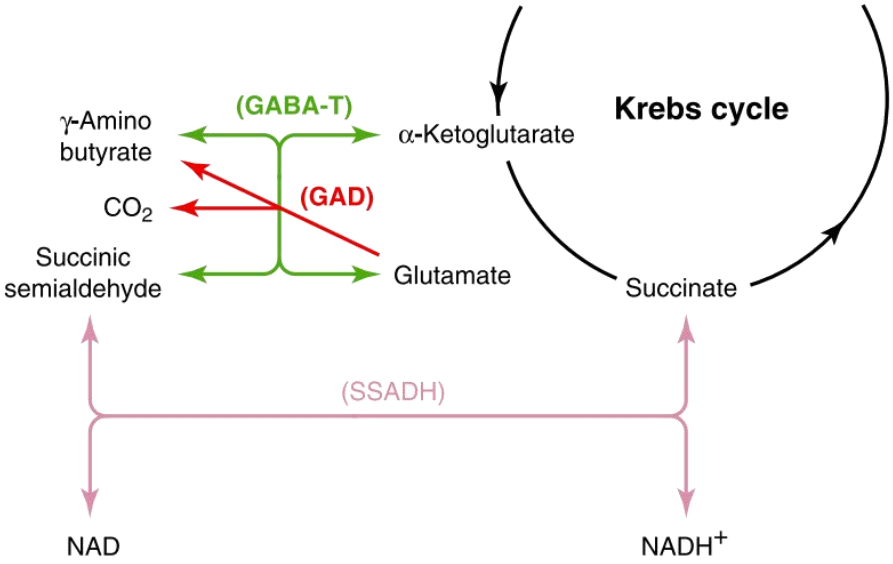


FIGURE 2.1 – GABA metabolism.

GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; NAD, nicotinamide adenine dinucleotide; SSADH, succinic semialdehyde dehydrogenase (Siegel *et al*, 2006).

2.1.2 $GABA_A$ receptors

2.1.2.1 Structure

GABA neuronal inhibition is mediated by two different receptors: type A receptors ($GABA_A$) and type B receptors ($GABA_B$) (***FIGURE 2.2***). $GABA_B$ receptors are coupled indirectly *via* G-proteins to either Ca^{2+} or K^+ channels to produce slow and prolonged inhibitory responses (Bowery, 2010). A third class designated $GABA_C$ was first identified in the vertebrates' retina (Chebib, 2004; Lukasiewicz *et al*, 2004), but they are now viewed as a variant of $GABA_A$ receptors exclusively composed of ρ subunits (see below). The Nomenclature Committee of the International Union of Pharmacology recommends to designate them as $GABA_A$-ρ receptors (Olsen and Sieghart, 2008).

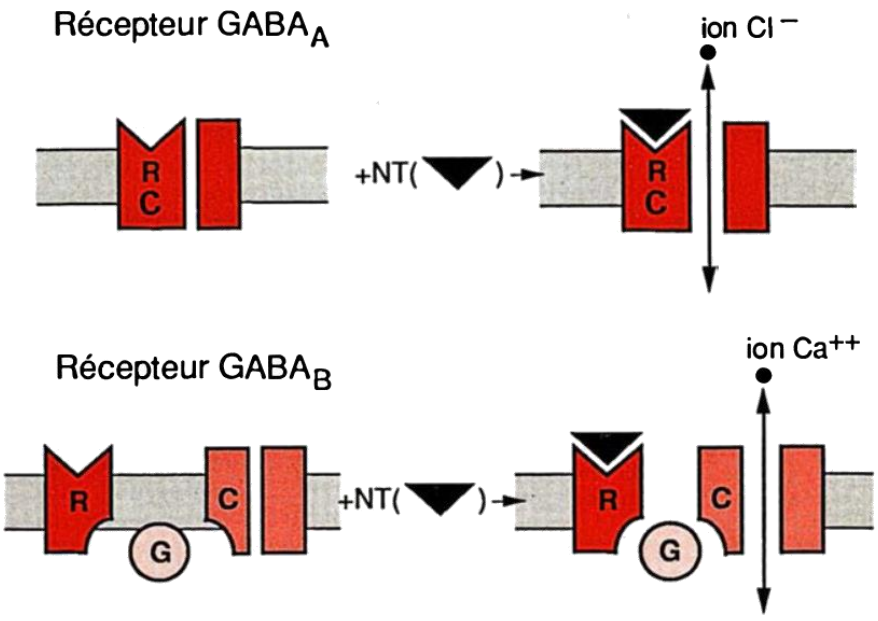


FIGURE 2.2 – GABA$_A$ and GABA$_B$ receptors.

GABA$_A$ receptors are channel receptors (RC) opening with the fixation of the neurotransmitter (NT), while GABA$_B$ receptors (R) are G-protein (G) coupled to an ion channel (C) (Bacon and Viennot, 1990).

GABA$_A$ receptors are hetero-oligomers composed of 5 protein subunits crossing the cellular membrane and forming a chloride (Cl^-) channel (***FIGURE 2.3***) (Olsen and Tobin, 1990). Binding of GABA on its site on the receptor opens the chloride ion channel, leading to a Cl^- influx according to the concentration gradient. Cellular membrane undergoes then an hyperpolarisation and thus a decreased excitability of the neuron (Siegel *et al*, 2006). Stabilising the membrane potential, GABA allows an upkeep of CNS functions, counterbalancing the effects of other excitatory neurotransmitters (e.g. glutamate). Bicuculline is the main GABA site antagonist of the GABA$_A$ receptors, but studies pointed out that it could also act as an allosteric inhibitor by binding to other sites of fixation that GABA could not reach (Johnston, 2013; Ueno *et al*, 1997).

The receptor subunits can be grouped in seven families, depending on their sequences. Some subunits are even composed of different variants, differing from one another in a few number of residues (Sieghart and Sperk, 2002). Thus, GABA$_A$ receptors are composed of 5 subunits amongst 19 (α_1–α_6, β_1–β_3, γ_1–γ_3, δ, ε, π, θ, and ρ_1–ρ_3). The majority of these receptors are assembled with the same base model: two α-subunits, two β-subunit and one γ-subunit

(Whiting, 2006). This combination is required for a fully functional $GABA_A$ receptor (Angelotti and Macdonald, 1993). $GABA_A$ receptors with the $\alpha_1\beta_2\gamma_2$ pattern thus account for 43% of these receptors in the mammalian brain (McKernan and Whiting, 1996; Sieghart and Sperk, 2002). The α-subunits are the most interesting, as they are both important for GABA (between α- and β-subunits) and benzodiazepines (between α- and γ-subunits) fixation on their respective sites on the receptor (***FIGURE 2.3***).

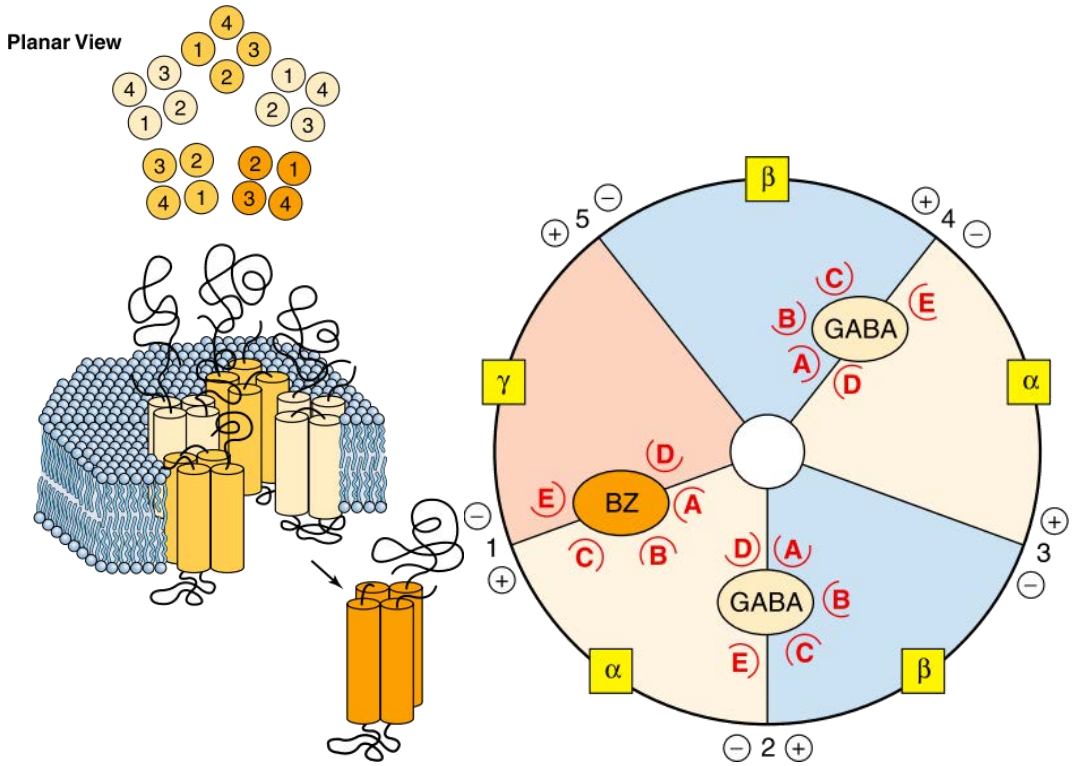


FIGURE 2.3 – Schematic representation of $GABA_A$ receptors.

A-E: peptides loops; BZ: benzodiazepine site (Olsen and Tobin, 1990; Siegel *et al*, 2006).

2.1.2.2 Brain location

While GABA synapses seem to be omnipresent in the mammalian brain, as they account for up to 30-40 % of the CNS synapses, some brain regions concentrate most of them. GABAergic neurons are thus mostly located in the cerebellar Purkinje cells and the hippocampal formation of rat brain (Radian *et al*, 1990). $GABA_A$ receptors are fairly widespread in the brain but the densities are particularly high in the cerebellum, the olfactory bulb, the thalamus, the

hippocampal formation and the cerebral cortex in rat brain (***FIGURE 2.4***) (de Blas *et al*, 1988; Bowery *et al*, 1987; Palacios *et al*, 1981; Young and Chu, 1990). The hippocampal formation and cerebral cortex regions are highly linked to anxiety regulation (cf. ***3.2.1.3*** and ***3.2.2.1***, respectively).

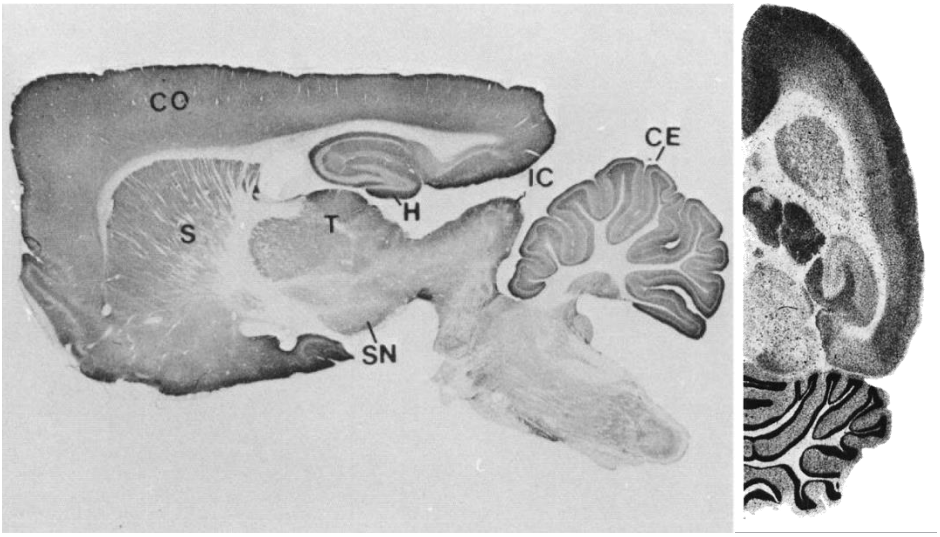


FIGURE 2.4 – Localisation of $GABA_A$ receptors in the rat brain.

Left: anti-GABA receptor antibody; right: [^{3}H]GABA autoradiography. CO, cerebral cortex; CE, cerebellum; H, hippocampal formation, IC, inferior colliculus; S, corpus striatum; SN, substantia nigra; T, thalamus (de Blas *et al*, 1988; Young and Chu, 1990).

It has since then been demonstrated that specific $GABA_A$ receptors, depending on their subunits composition, are differently expressed in brain regions (Fritschy and Mohler, 1995; McKernan and Whiting, 1996; Sieghart and Sperk, 2002). ***TABLE 2.1*** sums up the different major $GABA_A$ subtypes, depending on their subunits composition, and their major CNS location. It has also been pointed out that the expression of these subunits in the different brain regions vary during the development of the organism (Laurie *et al*, 1992).

TABLE 2.1 – Distribution of the major $GABA_A$ receptor subtypes in the rat brain.

(McKernan and Whiting, 1996; Uusi-Oukari and Korpi, 2010).

Subtype	Relative abundance in rat brain (%)	CNS location
$\alpha_1\beta_2\gamma_2$	43	Most brain areas. Interneurons in hippocampal formation, cerebral cortex and cerebellum
$\alpha_2\beta_{2/3}\gamma_2$	18	Spinal cord motoneurons, hippocampal formation
$\alpha_3\beta_n\gamma_{2/3}$	17	Cholinergic and monoaminergic neurons
$\alpha_2\beta_n\gamma_1$	8	Limbic system
$\alpha_5\beta_3\gamma_{2/3}$	4	Hippocampal formation
$\alpha_6\beta\gamma_2$	2	Cerebellum
$\alpha_6\beta\delta$	2	Cerebellum
$\alpha_4\beta\delta$	3	Thalamus and hippocampal formation
Other minor subtypes	3	Present throughout brain

As far as α-subunits are concerned, the α_1-subunit is the most widespread and ubiquitously distributed in the brain, whereas α_2-, α_3-, α_4-, α_5-, and α_6-subunits are confined to more specific brain regions (***FIGURE 2.5*** and ***TABLE 2.2***) (D'Hulst *et al*, 2009; Fritschy and Mohler, 1995; Sieghart and Sperk, 2002). Thus, the α_2-subunit is mostly located in the forebrain, with the highest concentration being in the olfactory bulb, striatum, accumbens nucleus, septum, hippocampal formation, amygdala, and hypothalamus. The α_3-subunit is present in the olfactory bulb, cerebral cortex, thalamus, and amygdala. The α_4-subunit was identified in the thalamus, hippocampal formation, and olfactory bulb. The α_5-subunit was detected in the hippocampal formation, olfactory bulb, and hypothalamus while α_6-subunit is only present in the cerebellum. To go further, specific regions, such as the amygdala, have also presented differences in terms of $GABA_A$ receptors α-subunits. Indeed, only the central nucleus revealed a great expression of the α_2-subunit, while the α_1-subunit was moderately expressed throughout the amygdala (Kaufmann *et al*, 2003).

The inhibitory properties of GABA neurotransmitter as well as the specific repartition of its $GABA_A$ receptors in the brain, make this GABAergic system particularly involved in the central regulation of anxiety.

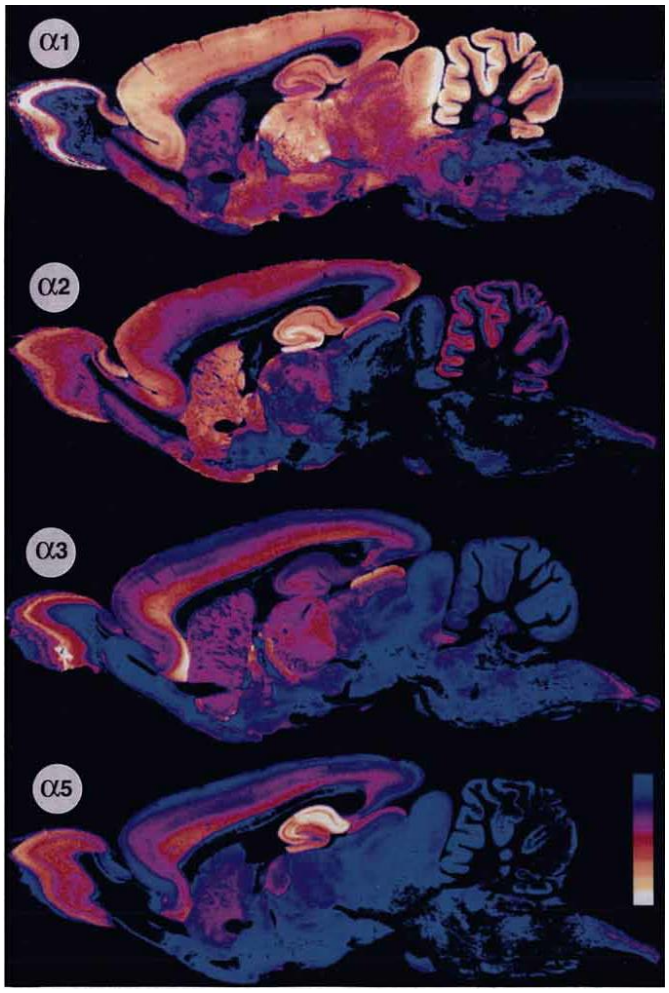


FIGURE 2.5 – Regional variations of the alpha subunits of $GABA_A$ receptors. Color-coded immunohistochemistry: the strongest signal is depicted in white while the background is in dark blue. 7, facial nucleus; ac, anterior commissure; AO, anterior olfactory bulb; cc, corpus callosum; CPu, caudate putamen; IC, inferior colliculus; Igr, internal granular layer of the olfactory bulb; Int, intercalated cerebellar nucleus; IO, inferior olive; LV, lateral ventricle; ml, medial lemniscus; Mo5, motor trigeminal nucleus, ox, optic chiasm; Pn pontine nucleus; Rt, reticular thalamic nucleus; SC, superior colliculus; scp, superior cerebellar peduncle; Th, thalamus; TT, tenia tecta; Tu, olfactory tubercle; VP, ventral posterior thalamic nucleus (Fritschy and Mohler, 1995).

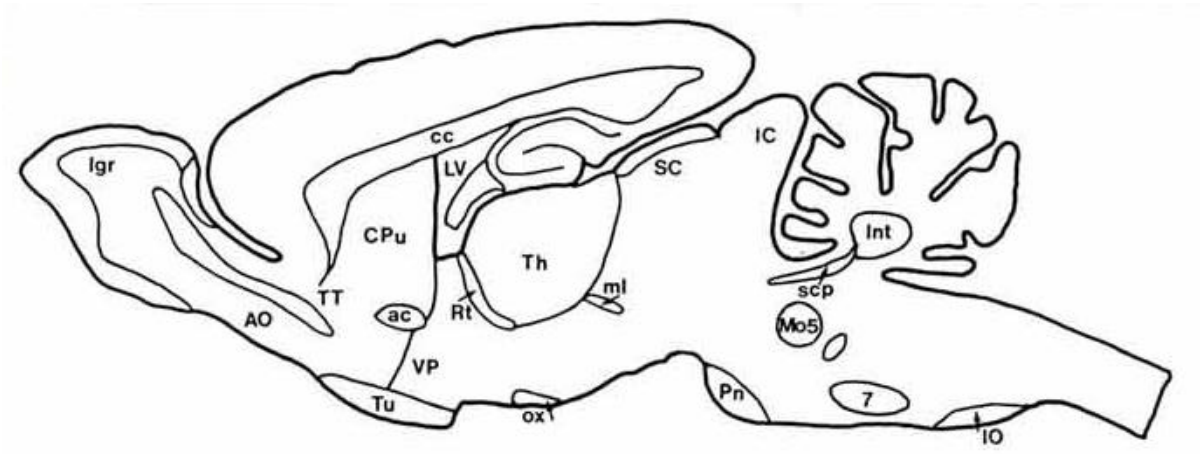


TABLE 2.2 – Brain location of the $GABA_A$ α subunits.

Adapted from (D'Hulst *et al*, 2009; Fritschy and Mohler, 1995; Uusi-Oukari and Korpi, 2010).

α subunit	High expression brain location	Effect
α_1	cerebral cortex, basal forebrain, pallidum, thalamus, tectum, cerebellum	- Mediation of sedative effect of diazepam - Mediation of amnesic action of diazepam - Mediation of anticonvulsant action of diazepam (partial)
α_2	olfactory bulb, cerebral cortex, hippocampal formation, amygdala, striatum, cranial nerve motor nuclei	- Mediation of anxiolytic action of diazepam - Mediation of myorelaxant action of diazepam (partial)
α_3	olfactory bulb, cerebral cortex, amygdala, septum, thalamus	Mediation of myorelaxant action of diazepam (partial)
α_4	olfactory tubercle, hippocampal formation, striatum, thalamus	N/A
α_5	olfactory bulb, hippocampal formation, spinal trigeminal nucleus	Mediation of myorelaxant action of diazepam (partial)
α_6	cerebellum	N/A

2.1.3 GABA and $GABA_A$ receptor: role in anxiety

Many links were made between GABA transmission and anxiety disorders (Kalueff and Nutt, 2007; Nuss, 2015). Here are a few examples.

Some anxiety-like behaviours are associated with a decreased GABA transmission. Thus, an increased GABAergic transmission in the amygdala leads to decreased anxiety-like behaviours and inversely (Davis *et al*, 1994; Shekhar *et al*, 2003). Similarly, glutamic acid decarboxylase (GAD, cf. ***2.1***) knockout mice displayed increased anxiety-like behaviours (Kash *et al*, 1999). Eventually, the transplant of GABAergic neural grafts directly in the lateral and basolateral amygdala nuclei decreased the anxiolytic-like behaviours of rats in the elevated plus-maze (Cunningham *et al*, 2009). In contrast with the previous observations, high-anxiety mice presented an imbalanced GABAergic neurotransmission with an enhanced GABA synthesis and release in the amygdala compared to low-anxiety mice, maybe as a compensatory mechanism of overstimulation of the limbic system (Tasan *et al*, 2011).

As far as $GABA_A$ receptors are concerned, 'handling-habituated' rats (handled four times a day, 6-8 consecutive days) exhibited a higher number of these receptors than 'naïve' rats (left in their home-cage for 6-8 days) (Biggio *et al*, 1990). It has then be hypothesised that 'handling-habituated' rats represented an unstressed group while the acute handling of 'naïve' rats before sacrifice caused an emotional distress. These results were confirmed with Cl^- influx: the handling habituated rats displayed a higher Cl^- influx in neurons from the cerebral cortex than 'naïve' rats. Eventually, it was also stated that high-anxiety selected rats (cf. ***4.1.2***) displayed a lower expression of $GABA_A$ receptors in the central amygdala compared to low-anxiety rats (Skórzewska *et al*, 2015).

2.1.4 $GABA_A$ receptors pharmacological modulation

It has also been discovered that GABA action on $GABA_A$ receptors can then be modulated by several other sites potentiating or inhibiting GABA action. Overall, due to its inhibiting effect on the CNS, an opening of the ion channel of $GABA_A$ receptors brings a Cl^- influx, inducing sedative, anxiolytic, anticonvulsant, muscle relaxant, and memory-impairing actions, while closing the Cl^- channel triggers stimulating, anxiogenic, convulsing, and promnesiant effects (Siegel *et al*, 2006).

The allosteric modulating sites are the following: benzodiazepine site, ethanol site, barbiturate site, neurosteroid site, and picrotoxin site (***FIGURE 2.6***). Many molecules have then be identified as allosteric modulators of the $GABA_A$ receptor by fixating to one of the previously mentioned sites (Möhler, 2011; Olsen, 2014, 2015). The benzodiazepine site and its specific role in anxiety will be detailed later in this section (cf. ***2.2.3***). Ethanol and barbiturates potentiate GABA action *via* their respective sites, thus having mostly sedative effects (including anxiolytic properties) (Olsen, 2014). More specifically, barbiturates allosterically enhance the binding of GABA and benzodiazepine, prolonging the opening of $GABA_A$ channel, while inhibiting the binding of $GABA_A$ antagonists (Macdonald and Olsen, 1994). Ethanol action on the receptor may vary from a brain region to another (Gray, 1982). The neurosteroid site is situated between the α- and the β–subunits, close to the chloride ion channel (Nuss, 2015). It has been demonstrated that progesterone potentiates the function of GABA, triggering sedative and anxiolytic properties, while other gonadal steroids (e.g. oestrogens) do not affect GABA function (Macdonald and Olsen, 1994; Wilson, 1996). Eventually, picrotoxin is a product from

plants that possesses the ability to noncompetitively block $GABA_A$ function, and thus mostly act as a convulsant (Olsen, 2015).

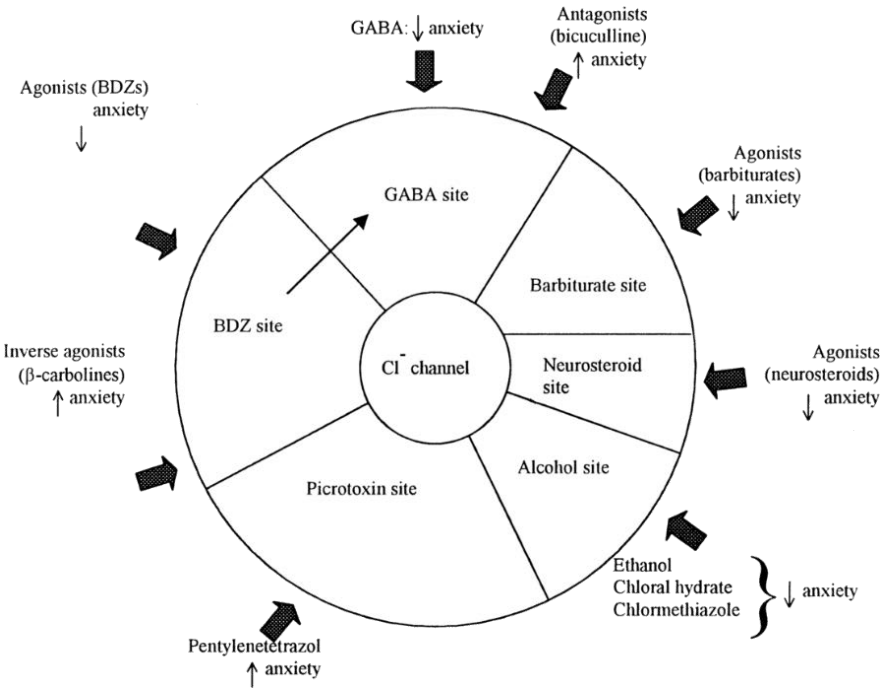


FIGURE 2.6 – Modulation by different molecules of the role of $GABA_A$ receptor in anxiety.

BDZ(s) : benzodiazepine(s) (Sandford *et al*, 2000).

The discovery of specific agonists/antagonists of these sites has led to the development of anxiolytic drugs to soothe anxiety disorders. Next section will specifically focus on the benzodiazepine family.

2.2 Associated anxiolytics: the benzodiazepines family

2.2.1 Chemistry

The discovery of **benzodiazepines** (BZDs) is an example of serendipity. It is the result of the work of Leo Sternbach, an Austro-Hungarian pharmacologist, who worked on quinolone/quinazoline derivatives for the manufacture of dyes. After his immigration during World War II to the United States, he started to work for Hoffman La Roche, where he had to find a molecule to replace meprobamate, the current anti-anxiety drug (cf. ***3.3.2***). Forced to stop his research, he put several of his samples aside. It was a few years later, with the work of a

colleague that strong calming properties were associated to one of the quinolone derivatives, combined with a low toxicity *in vivo*. Chlordiazepoxide (***FIGURE 2.8***) was thus discovered (and sold in 1960), followed some years later by diazepam (***FIGURE 2.8***, marketed in 1963) and both would revolutionise the anti-anxiety drugs market.

Chemically, these two molecules are derived from the same structure, the benzodiazepine ring that is the combination of a benzene and a diazepine ring. The diazepine is a heterocycle with two nitrogen and five carbon atoms and the maximum possible number of double bonds. Nitrogen atoms can be present in positions 1,4, 1,5 or 2,3 (***FIGURE 2.7***). Three classes of BZDs can then be discerned, but most prescribed benzodiazepines are the 1,4 due to their more interesting pharmacological properties (***FIGURE 2.8***).

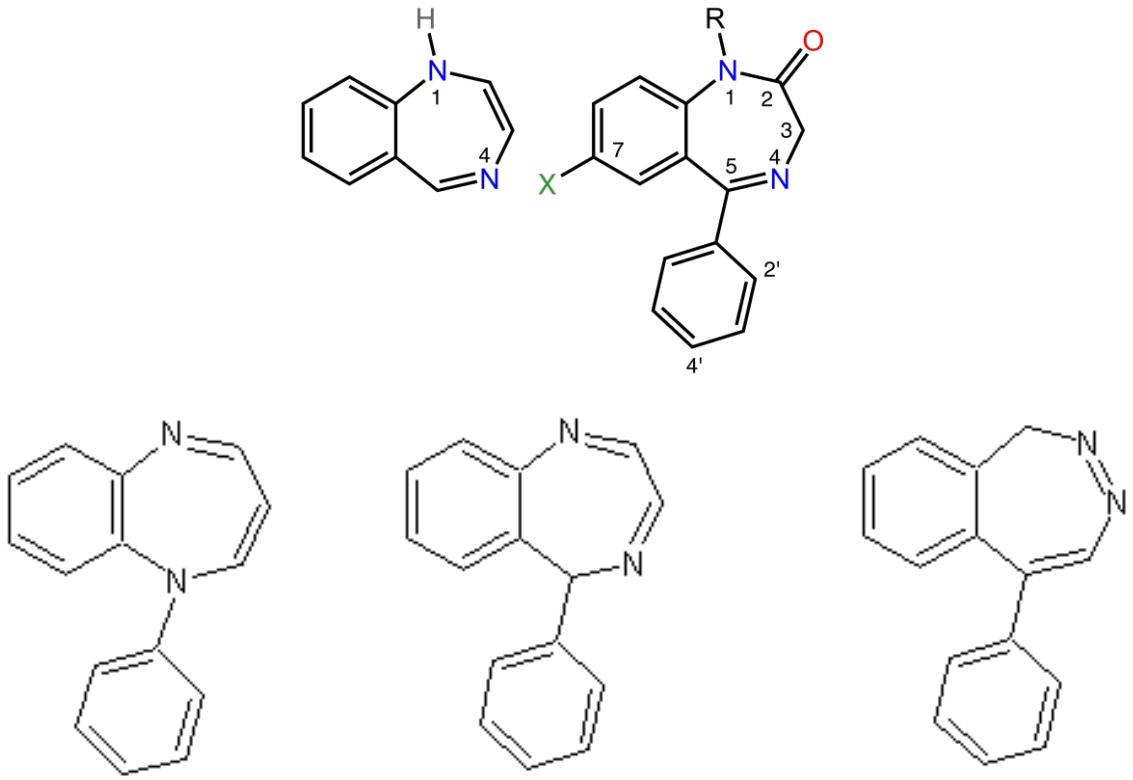


FIGURE 2.7 – Chemistry of benzodiazepines.

Top left: 1,4-benzodiazepine ring; top right: 5-phenyl-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one; bottom left: 1,5-benzodiazepine; bottom middle: 1,4-benzodiazepine; bottom right: 2,3-benzodiazepine.

Some substitutions can be performed to modify the pharmacological efficacy (***FIGURE 2.8***):

- Substitutions in the **seventh position** are the most common to increase the activity of the molecule: the electronegative properties of the substitute favour the binding to the BZD receptor (CF_3 > NO_2 > Br > Cl > OCH_3 > others); a NO_2 substitute triggers hypnotic properties to the molecule (e.g. flunitrazepam).
- Substitutions in the **first position** increase activity *via* an alkylation.
- Substitutes in the **second position** with an electronegative atom (O or N) were more common in the first generation of BZDs whereas more recent BZDs have fewer substitutions in this position.
- If there is no substitution in the **third position**, or if it is a hydroxyl functional group (OH, e.g. oxazepam), the polarity of the molecule is increased; a glucuronosylation in this position allows a faster elimination.
- Eventually, substitutions on the **benzene ring** may also take place: an ortho-substitution with a fluorine or a chlorine atom increase the activity of the molecule (electron-withdrawing group); another ring like a cyclohexenil ring can even substitute the benzene ring (e.g. in tetrazepam, a muscle relaxant BZD) or a pyridyl ring (e.g. in bromazepam).

The BZDs have basic properties, which allow them to bind to plasma proteins, favouring a fast blood transport. They are also very lipophilic, which allow them to cross the blood brain barrier, a common obstacle to the development of psychoactive drugs.

Some benzodiazepines-like molecules were also found in both cow and human milk (Medina *et al*, 1988; Peña *et al*, 1991). No further attempt to identify their origin and nature was yet made.

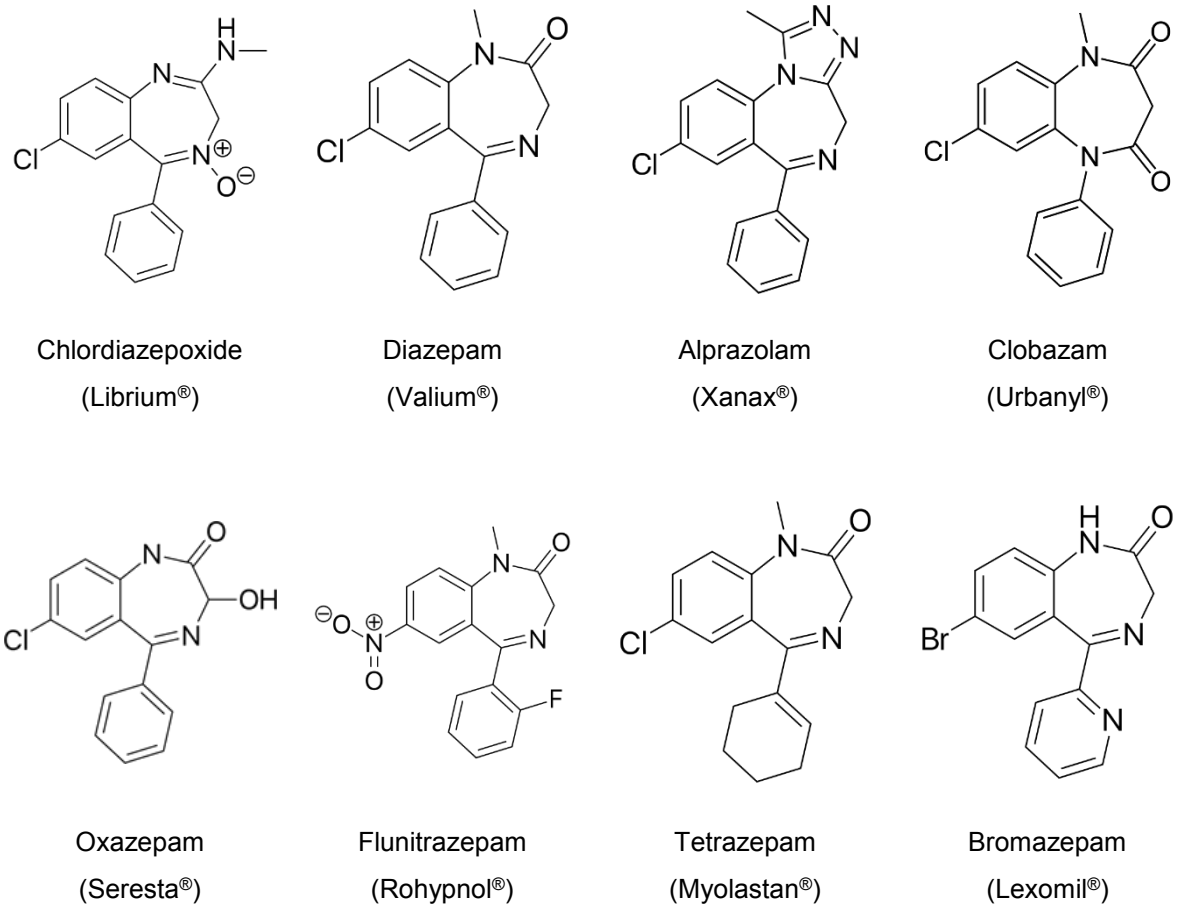


FIGURE 2.8 – Examples of benzodiazepines molecules.

Chlordiazepoxide: first BZD to be discovered (1960). Diazepam: second BZD to be discovered (1963); often used as the reference BZD in animal studies. Alprazolam: most sold BZD in France. Clobazam: an example of a 1,5 BZD. Oxazepam: an example of hydroxylation in the third position. Flunitrazepam: an example of NO_2 substitution in the seventh position (hypnotic BZD). Tetrazepam: an example of benzene ring substitution (muscle relaxant BZD). Bromazepam: an example of benzene ring substitution with a brome in seventh position.

2.2.2 Therapeutic effects

BZDs are psychoactive drugs and belong to the depressants family, which decrease the CNS activity and hence exert sedative effects. BZDs are then mostly prescribed for their anxiolytic, hypnotic, anticonvulsant and muscle relaxant properties, while they also exhibit amnestic effects.

Different forms of administration can be used. Oral administration ensures a quick action with an effect obtained a few hours after ingestion (***TABLE 2.4***). Effects are slower than oral administration *via* an intramuscular injection. Eventually, a rectal administration is used only when an intravenous injection cannot be performed: it is however optimal for a fast action in the CNS.

In 2013, twenty-two BZDs were commercialised in France and a few therapeutic effects are favoured depending on the molecule structure and half-life. The majority of them is prescribed for their anxiolytic and/or hypnotic properties (***TABLE 2.3*** and ***TABLE 2.4***) (ANSM, 2013). It is however sometimes hard to distinguish the two effects: indeed anxiolytic BZDs also triggers drowsiness while hypnotic BZDs are also known to be minor tranquilisers. Anxiolytic BZDs are prescribed to deal with the different anxiety-disorders described later (cf. ***3.3.1***) (Ashton, 1994).

TABLE 2.3 – Therapeutic effects of benzodiazepines.
(ANSM, 2013)

Action	Clinical use	Number of BZDs commercialised in France in 2013
Anxiolytic – inhibit anxiety	Anxiety disorders	11
Hypnotic – trigger sleep	Insomnia	7
Muscle relaxant – decrease muscle tone	Muscle spasms, pain, and hyperreflexia	2
Anticonvulsant – suppress excessive firing of neurons	Epileptic seizures	2
Amnesia – eliminate short-term memories	Premedication for medical operations, sedation for minor surgeries	0

Diazepam is on the *World Health Organization's List of Essential Medicines*, the most important medication needed in a basic health system, for both its anxiolytic and anticonvulsant properties (World Health Organization, 2015). Lorazepam and midazolam are also on the list for their anticonvulsant and sedative effects, respectively.

TABLE 2.4 – Anxiolytic BZDs in France.

(ANSM, 2013)

Molecule name	Common brand names	Year approved (France)	Time to peak (onset of action in hours)	Elimination half-life (hours)
Alprazolam	Xanax® & generic drugs	1982	1-2	10-20
Bromazepam	Lexomil® & generic drugs	1974	1-3	20
Chlordiazepoxide	Librax®	1988	1,5-4	5-30
Clobazam	Urbanyl®	1974	1-3	20
Clorazepate	Tranxene®	1974	Variable	30-150
Clotiazepam	Veratran®	1982	1-3	4
Diazepam	Valium®	1973	1-1,5	32-47
Ethyl ioflazepate	Victan®	1980	2,5-3	77
Lorazepam	Temesta® & generic drugs	1977	2-4	10-20
Nordiazepam	Nordaz®	1984	?	30-150
Oxazepam	Seresta® & generic drugs	1968	3-4	8
Prazepam	Lysanxia® & generic drugs	1975	2-6	30-150

Benzodiazepines anxiolytic effects were also unearthed in several animals' models due to the predictive criteria which was used to develop these tests (cf. ***4.1.1***), e.g. elevated plus-maze (Handley and Mithani, 1984), light-dark box (Crawley, 1981), open-field (Prut and Belzung, 2003) or conditioned defensive burying (Treit *et al*, 1981).

2.2.3 Mechanism of action

The receptors for BZDs are exclusively located in the CNS at the synaptic level and their pharmacological efficiency is dependent on the affinity of BZDs for these receptors (Möhler and Okada, 1977). Later, these receptors for BZDs have been shown to co-localise with the $GABA_A$ receptors, before the identification of a specific site directly on the $GABA_A$ receptor (Schoch *et al*, 1985; Sieghart and Sperk, 2002). The BZDs act as allosteric modulators of the $GABA_A$ receptor. The BZDs link to a site different from the GABA site and then enhance the affinity of GABA and increase the probability of the chloride channel to open, resulting in an increase influx of chloride ions and thus an hyperpolarisation of the neuron (***TABLE 2.5***) (Costa and Guidotti, 1979; Millan, 2003). The facts that GABA is needed for BZDs to exert their effect may explain the lesser toxicity of these drugs compared to other $GABA_A$ receptor ligands such as ethanol or barbiturates which directly act with the chloride channel (Gray, 1982).

TABLE 2.5 – Schematic representation of the effects of a benzodiazepine site agonist and an antagonist on $GABA_A$ receptor function.

(Atack, 2009).

GABA +	**No BZD ligand**	**Full agonist**	**Antagonist**
Example	/	Diazepam	Flumazenil
Outside cell Cl^- influx ↓ Inside cell			
Resting potential	–60 mV	–80 mV	–60 mV
Neuronal excitability	Normal	Hyperpolarisation = Less excitable	Normal

The search for endogenous ligands of this site finally led to the discovery of putative endozepines, molecules with an benzodiazepine ring, extracted from animal brains (Medina *et al*, 1988). These endozepines were found unevenly disseminated in brain, the highest concentration being spotted in the septum, amygdala, and hippocampal formation (Basile, 1991; Wolfman *et al*, 1991) but a possible contamination of external BZD molecules was raised (Farzampour *et al*, 2015).

BZD site antagonists (e.g. flumazenil) have little to no clinical effects as they only impede the fixation of BZDs on their receptors (***TABLE 2.5***) (Bentué-Ferrer *et al*, 1996). It is specifically used for BZD overdose ("Treatment of benzodiazepine overdose with flumazenil. The Flumazenil in Benzodiazepine Intoxication Multicenter Study Group.," n.d.; Weinbroum *et al*, 1996).

As previously skimmed through, $GABA_A$ receptors α-subunits may not relay in a similar manner the different therapeutic effects of BZDs (***TABLE 2.6***, cf. ***2.2.2***). The presence of both an α and a γ_2 ($\alpha_n\beta_n\gamma_2$ pattern receptor) is essential to convey the classical BZD-binding site (Angelotti and Macdonald, 1993; Fritschy and Mohler, 1995; Pritchett *et al*, 1989). $GABA_A$ receptors containing α_4- and α_6-subunits displayed a very low affinity for classical BZDs (Fritschy and Mohler, 1995; Lüddens *et al*, 1990; Wisden *et al*, 1991). While α_1-containing $GABA_A$ receptors mediate specifically the sedative, amnestic, and, in a lesser way, anticonvulsant effects of BZDs (Fradley *et al*, 2007; McKernan *et al*, 2000; Rudolph *et al*, 1999), α_2-containing $GABA_A$ receptors mediate the anxiolytic effects of benzodiazepines (Möhler, 2012; Morris *et al*, 2006; Rudolph and Möhler, 2006; Smith and Rudolph, 2012). Indeed, administration of diazepam did not induced anxiolytic properties in mice lacking the α_2-subunit (Dixon *et al*, 2008). This subunit also have a lesser role in carrying the anticonvulsant effects of diazepam (Fradley *et al*, 2007). The role of α_3-subunits in the anxiolytic effects of BZDs is more controversial (Rudolph and Knoflach, 2011). Indeed, the administration of diazepam in α_3-KO mice still induced anxiolytic properties (Löw *et al*, 2000), but a specific agonist of α_3-subunits containing $GABA_A$ receptors displayed anxiolytic-like effects in rats (Dias *et al*, 2005).

TABLE 2.6 – The effects of benzodiazepines on select $GABA_A$ receptors' α subunits.
Adapted from (Nutt, 2006; Rudolph *et al*, 1999).

Effects of benzodiazepines	α_1	α_2	α_3	α_4
Sedation	+	–	–	–
Anxiolysis	–	+	+/–	–
Amnesia	+			+
Myorelaxation	–		+	
Anticonvulsant	+	+	–	–

BZDs receptors are widely located in the rodent brain (Richards and Möhler, 1984; Schoch *et al*, 1985). Regions showing high densities of $GABA_A$ receptors modulated by BZDs are the cerebral cortices, cerebellum, amygdala, hippocampal formation, hypothalamus and accumbens nucleus (Richards and Möhler, 1984).

A few studies have identified the brain regions involved in the anxiolytic effects of diazepam. De Medeiros and colleagues identified that after a restraint stress in rats, diazepam-treated animals displayed a decreased neuronal activity in the cerebral cortex and the hippocampal formation and an increased neuronal activity in the central nucleus of the amygdala compared to the control group (de Medeiros *et al*, 2005). After having been placed in an elevated plus-maze, the diazepam-treated rats exhibited only an increased neuronal activity in the central nucleus of the amygdala and no effect in the other studied brain regions (accumbens nucleus and hippocampal formation) have been observed compared to the control group (Panhelainen and Korpi, 2012). Finally, the anxiolytic effects of diazepam after a social stress in rats were coupled with a decreased neuronal activity in several brain regions involved in anxiety regulation (cerebral cortex, accumbens nucleus, hippocampal formation, and hypothalamus) and an increased activity in the central nucleus of the amygdala compared to the control group (Lkhagvasuren *et al*, 2014). Overall, despite some differences due to the different models used or doses injected, diazepam decreased neuronal activity in the brain regions implicated in anxiety but increased neuronal activity specifically in the central nucleus of the amygdala, a sub-nucleus of the amygdala (cf. ***3.2.1.1***).

Some studies were also conducted with humans, using different approaches. Lorazepam decreased neuronal activity in the cerebral cortex (prefrontal and cingulate cortices) during an anxiety challenge (anticipation of aversive electrical stimulations) in a functional magnetic resonance imaging (fMRI) study (Schunck *et al*, 2010). In the same way, triazolam, an hypnotic BZD, decreased neuronal activity in cerebral cortex (prefrontal and cingulate cortices) and the hippocampal formation during an episodic memory challenge (orthographic categorisation) in positron emission tomography (PET) studies (Mintzer *et al*, 2001, 2006).

However, the 'GABA hypothesis' on anxiety regulation suffers from different limits. GABA neurons and $GABA_A$ receptors are indeed far too widely distributed in the brain (cf. ***2.1.2.2***), and all drugs enhancing GABAergic transmission do not act as anxiolytics (e.g. muscimol). Moreover BZD receptors are highly found in the cerebellum and the spinal cord, which are not directly related to anxiety regulation (Richards and Möhler, 1984). The question is then raised to know whether the anxiolytic effects of BZDs are actually a side effects of the common sedative and anticonvulsant effects of all $GABA_A$ ligands (Gray, 1982).

2.2.4 Side effects and pitfalls

BZDs are well-known for their numerous side effects (Griffin *et al*, 2013; O'brien, 2005; Stewart and Westra, 2002; Vgontzas *et al*, 1995). The daytime sedation (drowsiness, lethargy, and fatigue) is directly linked to the action of BZDs on $GABA_A$ receptors, especially the one located in the cerebellum (cf. ***2.2.2*** and ***2.2.3***). A numerous number of cognitive side effects (inattention, amnestic effects, and disinhibition) can also be linked to this action (Buffett-Jerrott and Stewart, 2002).

Beyond these specific side effects, BZDs also share pharmacological dependence (Bateson, 2002; Podhorna, 2002) and addiction (Tan *et al*, 2010) with other drugs that target the nervous system. Pharmacological (or physiological) dependence and addiction are often being confused, the former being typical of the latter despite not being a required criterion. Pharmacological dependence is a natural physiological adaptation in response to the consumption of many type of drugs (triggering both tolerance and withdrawal), whereas addiction (called substance abuse in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders [DSM-V]) implies compulsive use and drug-seeking behaviours, as well as tolerance and withdrawal symptoms, amongst others (American Psychiatric Association, 2013; O'brien, 2005).

Some clinical studies also spotted an association between the consumption of BZDs and the risk of dementia (Billioti de Gage *et al*, 2014), but these results are being challenged by other studies (Lagnaoui *et al*, 2009) or specifically associated to the consumption of long half-life BZDs (e.g. diazepam) (Shash *et al*, 2016). French Agency for the Safety of Health Products (*Agence Nationale de Sécurité du Médicament et des produits de santé*, ANSM) has stated that the use of BZDs amongst elderly (over 65 years old) living alone is associated with an increased risk of dementia (ANSM, 2013).

Despite their invaluable use as a therapeutic treatment for anxiety disorders, BZDs also have a potential of abuse, often used in association with other drugs to get high, triggering both pharmacological dependence and addiction (O'brien, 2005). BZDs were thus ranked 7th in terms of dependence, physical arm, and social harm in comparison with 20 popular recreational drugs (Nutt *et al*, 2007).

However, despite all these side effects BZDs are still the leading treatment for anxiety disorders, especially in France (ANSM, 2013; Stahl, 2002) (cf. ***3.3.2***).Some new anxiolytics are then needed to overcome BZDs' side effects. Amongst the different potential molecules that might help replace BZDs, bioactive peptides, or foods containing bioactive peptides, are of special interest, due to their lesser potential side effects and toxicity.

2.3 The α_{s1}-casein tryptic hydrolysate (CH)

2.3.1 Biological properties in rodents: benzodiazepine-like profile

A **tryptic hydrolysate of bovine α_{s1}-casein (CH)**, intraperitoneally (i.p.) injected (3 mg/kg), displayed anxiolytic-like properties in rats (Miclo *et al*, 2001) in two different rodent's behavioural models of anxiety (the elevated plus-maze and the conditioned defensive burying tests, cf. ***4.1.1***). These results were confirmed after an oral administration of the tryptic hydrolysate (iCH) industrial formula (Lactium®, Ingredia, France) in rats (minimal dose: 15 mg/kg) in both elevated plus-maze and conditioned defensive burying models (Violle *et al*, 2006).

Associated to these anxiolytic-properties, iCH also exerts some positive effects on sleep. Indeed, an oral administration of iCH (15 mg/kg) prevented stress-induced sleep disturbance in

rats: the slow wave sleep time was preserved and the period of paradoxical sleep was slightly improved (Guesdon *et al*, 2006). These effects were also observed in mice: an oral administration of iCH (150 mg/kg) enhanced the sleep induced by pentobarbital sodium (42 mg/kg, i.p.) by increasing duration of sleep but not the onset of sleep (Dela Peña *et al*, 2016).

Eventually, an i.p. injection of CH (3 mg/kg) inhibited the seizures induced by an i.p. injection of pentylenetetrazole (60 mg/kg) in a rat model (Miclo *et al*, 2001).

2.3.2 Security profile: absence of benzodiazepines' side effects

As shown overhead, these effects are comparable to that of diazepam; CH may thus have some BZD-like properties. A screening for any side effect was then carried out.

Compared to diazepam, iCH, orally administered in a rat model (15 mg/kg), displayed neither tolerance (seven days of administration, conditioned defensive burying on the 8th day), nor memory-impairing effects (passive avoidance), nor addiction (conditioned place preference) (Messaoudi *et al*, 2009). The absence of sedation was also characterised in mice after an oral administration (150 to 500 mg/kg) in both open-field (Hall and Ballachey, 1932) and rotarod performance (Dunham and Miya, 1957) models (Dela Peña *et al*, 2016).

2.3.3 Use in veterinary medicine

The anxiolytic-like properties of iCH were then tested within other mammalian species in order to apply the effects of the hydrolysate in veterinary medicine.

An oral supplementation (15 mg/kg) of iCH for 56 days amongst 34 cats significantly reduced the global score of anxiety (more specifically fear of strangers, contact with familiars, general fears, fear-related aggressions and autonomic disorders) compared to a placebo treatment (Beata *et al*, 2007a). The study was reiterated amongst 38 dogs with an oral supplementation (15 mg/kg) during 56 days. At the end of the treatment period, iCH reduced the anxiety score amongst dogs and no difference was observed compared to selegiline, a specific inhibitor of monoamine oxidase, used as a reference molecule to soothe

hypersensitivity, separation anxiety or phobias (Beata *et al*, 2007b). Eventually, the efficacy of a 65-days access to a diet containing iCH on behavioural and physiological parameters was evaluated amongst two groups of Beagle dogs: 16 anxious dogs and 16 non-anxious dogs. At the end of the intervention, the iCH supplementation positively impacted the behavioural observations and significantly decreased plasma cortisol level in the anxious dogs group (Palestrini *et al*, 2010).

Effects of iCH were also evaluated amongst 6 semi-feral ponies on training efficiency during transition to domestic management and handling (McDonnell *et al*, 2013). Half of them received an oral supplementation of iCH (1000 mg/day for a weight between 160 and 205 kg) during two weeks, starting 5 days before the transition. Compared to placebo (oat flour), iCH-treated ponies displayed better behavioural scores during the possibly stressful circumstances of domestic training. A second study was performed on 10 horses showing aversion to specific healthcare procedures (McDonnell *et al*, 2014). Half of them received an oral iCH supplementation (2000 mg/day for a weight ranging from 450 to 600 kg) for 5 days. Behaviours were scored at the end of the treatment period, and iCH-treated horses displayed an improvement for seven of the ten aversions studied, displaying a modest benefit effect of iCH on slightly aversive routine healthcare actions.

2.3.4 Clinical studies: use as a food supplement

The next step was then to investigate the anxiolytic-like properties of iCH amongst humans. A first double-blind study (Lanoir *et al*, 2002) was carried out on 72 women, half of them receiving 150 mg/kg of iCH during 30 days. Heart rate and blood pressure were evaluated after 10 and 30 days of treatment, using the Stroop test as a psychological stressor. iCH decreased the increase of blood pressure induced by the test amongst more anxious individuals.

A second double-blind study (Messaoudi *et al*, 2005) assessed the anxiolytic-like effect of an oral supplementation of iCH (400 mg) compared to a placebo (bovine skimmed milk powder, 400 mg) amongst 42 men exposed to two types of stressors on hemodynamic and cortisol responses. The two stressors were a psychological one (Stroop test, ***FIGURE 2.9***) and a physical one (cold pressor test, ***FIGURE 2.10***). The supplementation was given twice the day before the experiment (at 08:00 a.m. and 07:00 p.m.) and the day of the experiment at 08:00 a.m. Compared to the placebo, iCH decreased the augmentation of blood pressure and also decreased

plasma cortisol concentration in men exposed to both stressors. As no changes in reactivity, arousal, heart rate and ambulatory blood pressure were observed, it was stated that iCH was not a β–blocker, nor a hypotensive agent.

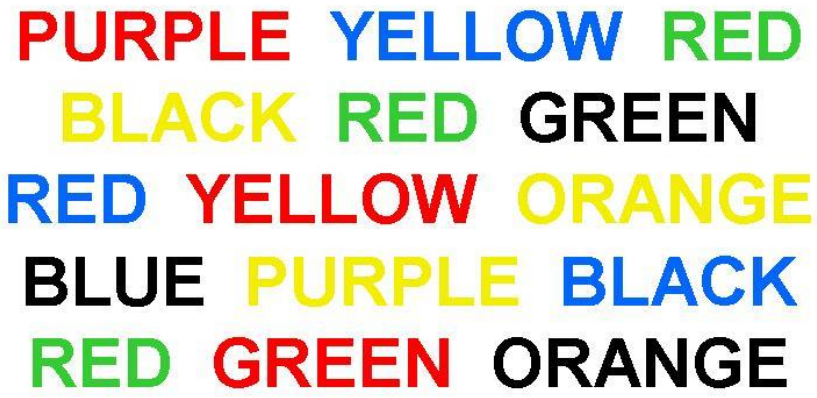


FIGURE 2.9 – A Stroop test example.

This test is based on the fact that naming the colour of a word takes longer and is more prone to errors when the colour of the ink does not match the name of the colour. The goal of this test is then to name the colour in which the word is written and not the word itself. As an example, the first word above must be read “red” and not “purple”. This test is then frequently used in psychological studies, to evaluate attention, but can also be used to trigger stress responses amongst individuals, especially when signalling the errors to the patient (Renaud and Blondin, 1997).

A third double-blind cross-over study (Kim *et al*, 2007) was realised amongst 63 women displaying stress symptoms. Women were given either iCH (150 mg/day) or placebo (skimmed milk power, 150 mg/day) during a first 30 days period, followed by a three weeks-washout at the end of which treatments were switched during 30 days. Self-reported questionnaires evaluated the impact of both treatments on physical (digestive tract, respiratory system, cardiovascular system, locomotor system, other physical symptoms of stress) and psychological (intellectual functions, emotional area) area impacted by stress on 0, 15 and 30 days after the start of each interventional period.

FIGURE 2.10 – The cold pressor test.

This is a cardiovascular test that consists of putting the hand of the patient into slushy ice water. The temperature raises both blood pressure and heart rate. If used more than 1 minute, this test can trigger stress responses and is sometimes also even used as a pain tolerance test.

Eventually, a clinical double-blind study evaluated the effect of a supplementation TARGET 1® composed of iCH associated with taurine, *Eleuthrococcus senticosus* (siberian ginseng) and Extramel® (a proprietary melon juice concentrate) on burnout symptomatology amongst 87 participants (Jacquet *et al*, 2015). Participants received a 12 weeks supplementation of either TARGET 1® (1280 mg/day, containing 150 mg of iCH) or placebo. TARGET 1® significantly improved the symptoms of participants after a 12 weeks supplementation.

After some rejects from the French Food Safety Agency (Agence Française de Sécurité Sanitaire des Aliments, Afssa) (Hirsch, 2001, 2002, 2003, 2005), Lactium® (iCH) finally got the claim "can moderate the blood pressure response induced by stress amongst women who are particularly sensitive"[2] (Briand, 2007). The American Food and Drug Administration (FDA) recognized Lactium® as Generally Recognised as Safe (GRAS) and New Dietary Ingredient (NDI) (FDA, 1997), while in 2009 the Australian Therapeutic Goods Administration registered Lactium® on the Australian Register of Therapeutic Goods. Biological effects of Lactium® were also recognized by Korea Food and Drug Administration (KFDA).

[2] '*peut modérer la réponse tensionnelle au stress chez les femmes qui y sont particulièrement sensibles*'

2.4 α-casozepine, a decapeptide carrying the anxiolytic-like effects of iCH

2.4.1 *In vitro* discovery, *in vivo* properties

The BZD-like profile of CH and/or iCH (anxiolytic-like, sleep-modulating and anticonvulsant properties, cf. ***2.3.1***) led to the hypothesis that one or more of the peptides composing CH may have an affinity for BZD site on $GABA_A$ receptors.

The different peptides composing CH were then screened on their ability to displace [methyl-^{3}H]-flunitrazepam of the BZD site of $GABA_A$ receptors (Miclo *et al*, 2001). CH displayed an affinity for the BZD site of $GABA_A$ receptor (IC_{50} = 72 µM). In the exact same conditions, a ten-residue peptide, corresponding to the 91-100 fragment of α_{s1}-casein (1.8% of Lactium® and about 5% of CH), also displays an affinity for the BZD site (IC_{50} = 88 µM). The peptide was named **α-casozepine** (α-CZP) and its sequence was found to be YLGYLEQLLR (1266.6 Da). In the same conditions, the IC_{50} of diazepam was 8.2 nM, α-CZP showing then a 10,000 times lower affinity for the BZD site of $GABA_A$ receptor than diazepam. A synthetic α-CZP was also tested, but was four times less potent than the natural one (IC_{50} = 370 µM). None of the other peptides composing CH had affinity for the BZD site of $GABA_A$ receptor.

A study of the conformation of α-CZP using ^{1}H-NMR, revealed that the peptide adopts an rigid helicoid structure in water/SDS micellar medium, while the relative disposition of the tyrosine aromatic rings and the distance between their both centres were similar to those of the aromatic rings in nitrazepam, a BZD (***FIGURE 2.11***) (Lecouvey *et al*, 1997). This might explain the affinity of the peptide for BZD site of $GABA_A$ receptor.

The anxiolytic-like properties of α-CZP were then assessed using the conditioned defensive burying test in a rat model (Miclo *et al*, 2001). An i.p. injection of the peptide (0.4 mg/kg) displayed anxiolytic-like effects compared to vehicle, which were similar to those of diazepam (1 mg/kg, i.p.). α-CZP might then be the carrier of CH anxiolytic-like properties. Anxiolytic-like properties were also confirmed after an i.p. injection (3.0 to 12.6 mg/kg) or an oral administration (4.4 mg/kg) in a mice model, using the elevated plus-maze (Mizushige *et al*, 2013b).

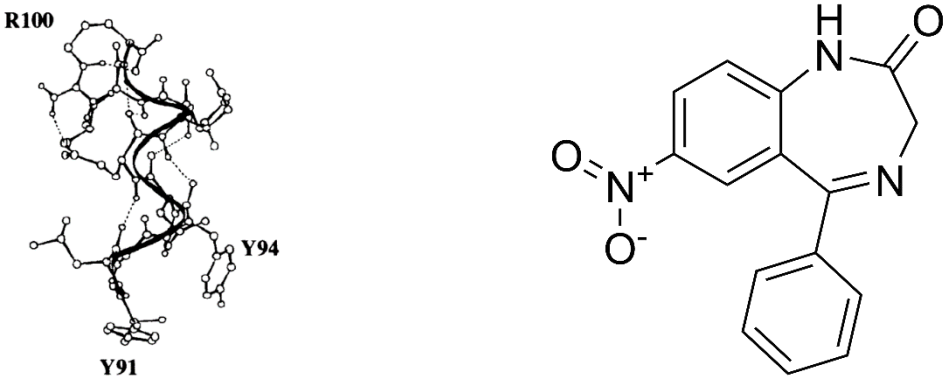


FIGURE 2.11 – Comparison of the time-average structure from the last 30 picoseconds of restained-molecular dynamics simulation of α-CZP with the structure of the benzodiazepine nitrazepam.
Structure of α-casozepine. Aromatic rings of residues of tyrosine 91 and 94 should be compared with the two aromatic rings of nitrazepam (Lecouvey *et al*, 1997).

2.4.2 α-casozepine derivatives

As the intact sequence of α-CZP seemed to be necessary to display the anxiolytic-like activity, *in vitro* digestibility of α-CZP was assessed to investigate the stability of the peptide in the gastro-intestinal tract (Cakir-Kiefer *et al*, 2011b). A set of different proteases combination was chosen: pepsin, chymotrypsin/trypsin, Corolase PP^{TM}, pepsin followed by chymotrypsin/trypsin or pepsin followed by Corolase PP^{TM}. The bonds present in the 91-95 fragment were resistant to hydrolysis induced by the combinations cited overhead. The 91-97 fragment (α_{s1}-CN-(f91–97), YLGYLEQ) as well as the 91–95 fragment (α_{s1}-CN-(f91–95), YLGYL) were found in significant amount within all hydrolysis media (Balandras *et al*, 2008, 2009). The anxiolytic-like properties of α_{s1}-CN-(f91–97) were assessed in rats after an i.p. injection (0.5 mg/kg) in conditioned defensive burying, elevated plus-maze and light/dark box. The anxiolytic-like effects of α_{s1}-CN-(f91–97) were similar to that of diazepam (1 mg/kg, i.p.) and α-CZP (0.7 mg/kg, i.p.). This fragment may then be the *in vivo* carrier of α-CZP anxiolytic-like activity.

The *in vitro* absorption of α-CZP and f91–97 was then evaluated using a Caco-2 cells monolayer to mimic the intestinal epithelium, with different preparations of bile salts (porcine bile extract; equimolar mixture of taurocholate, cholate and deoxycholate) in a view to approximate physiological conditions (Cakir-Kiefer *et al*, 2011a). In the presence of bile salts, the N-terminal part of both α-CZP and α_{s1}-CN-(f91–97) was resistant to brush-border peptidases. On the other hand, the C-terminal part of α-CZP was hydrolysed, leading to the formation of α_{s1}-CN-(f91–97). The bile salts also increased the apparent permeability coefficient of both α-CZP and α_{s1}-CN-(f91–97), the latter being more absorbed than α-CZP.

2.4.3 Other anxiolytic peptides derived from α-casozepine

Other peptides derived from α-CZP may also be the carrier of α-CZP action.

YLG, α_{s1}-CN-(f91–93), exhibits anxiolytic-like activity in the elevated plus-maze in a mouse model after either an i.p. injection (1 mg/kg) or an oral administration (1 mg/kg) (Mizushige *et al*, 2013b). This tripeptide did not have any sedative effects in the open-field test after an i.p. injection (0.1 mg/kg).

It was also stated that the dipeptide YL, α_{s1}-CN-(f91–92), displayed anxiolytic-like effects after an i.p. injection (minimum 0.1 mg/kg), an oral administration (minimum 0.3 mg/kg) or an intracerebroventricular injection (0.1 nmol/mouse) in a mice model using elevated plus-maze (Kanegawa *et al*, 2010). YL did not exhibit sedation in the open-field test after an i.p. injection (0.1 mg/kg). Results of the anxiolytic-like properties of YL were confirmed after an oral administration (1 mg/kg) in a mice model using the elevated plus-maze test (Mizushige *et al*, 2013a, 2013b).

These results were widened to all aromatic amino acid-leucine dipeptides (Mizushige *et al*, 2013a). FL and WL also exhibited anxiolytic-like properties in the elevated plus maze after either an i.p. injection (1 mg/kg) or an oral administration (1 mg/kg), while LF and LW did not have any effect i.p. injected (1 mg/kg) compared to the vehicle. FL and WL also did not have any sedative effects in the open-field test.

2.4.4 A potential central mode of action of α-casozepine

Since the discovery of α-CZP's affinity for BZDs site of $GABA_A$ receptors, different attempts to understand the mode of action of the peptide were conducted. A central activity is privileged as α-CZP do not have any affinity for the peripheral BZD receptor (Miclo *et al*, 2001). It was also stated that iCH (which contains α-CZP) increased Cl^- influx in neuroblastoma cell culture in a dose-dependent manner (Dela Peña *et al*, 2016). This influx was blocked by bicuculline, a $GABA_A$ antagonist (Johnston, 2013), which goes in the same direction as α-CZP has affinity for $GABA_A$ receptors.

The involvement of different neurotransmitter systems (GABAergic, serotoninergic and dopaminergic) in the mode of action of α-CZP or some of its derivatives was also assessed, using different receptors ($GABA_A$, serotoninergic $5\text{-}HT_{1A}$ and dopamine D_1 receptors) antagonists. Bicuculline was used as a $GABA_A$ antagonist (Johnston, 2013), WAY100135 as a $5\text{-}HT_{1A}$ antagonist (Fletcher *et al*, 1993) and SCH23390 as a D_1 antagonist (Hyttel, 1983). α-CZP (10 mg/kg), YLG (3 mg/kg), YL (1 mg/kg), FL (1 mg/kg) and WL (1 mg/kg) anxiolytic-like activities induced by an i.p. injection in a mice model of elevated plus maze, were blocked by i.p. injection of bicuculline, WAY100135 or SCH23390 (5 mg/kg , 10 mg/kg and 30 µg/kg respectively) (Kanegawa *et al*, 2010; Mizushige *et al*, 2013a, 2013b). YL anxiolytic-properties were further blocked by an i.p. injection of flumazenil (1 mg/kg), a BZD site antagonist (Bentué-Ferrer *et al*, 1996). This indicates that at least $GABA_A$, $5\text{-}HT_{1A}$ and dopamine D_1 receptors are implicated in these peptides anxiolytic-like activities. Affinity of YLG, YL, FL and WL at 100 µM for all these receptors was negligible (Kanegawa *et al*, 2010; Mizushige *et al*, 2013a, 2013b).

Eventually, a mode of action was proposed for YL (Kanegawa *et al*, 2010). Different agonists for $GABA_A$, serotonin $5\text{-}HT_{1A}$ and dopamine D_1 receptors (muscimol, 8-OH-DPAT and SKF38393) were used in the elevated plus-maze, combined with the antagonists cited above. The following hypothesis was then formulated to explain the anxiolytic-like effect of YL: YL → serotonin release → $5\text{-}HT_{1A}$ receptor → dopamine release → D_1 receptor → GABA release → $GABA_A$ receptor → anxiolytic-like activity (***FIGURE 2.12***). The main limit in this model is that no link between YL and serotonin is actually made yet. Moreover, these receptors are broadly located in the brain and non-specifically implicated in anxiety. An injection of YL with different combinations of antagonists would have given a more specific model.

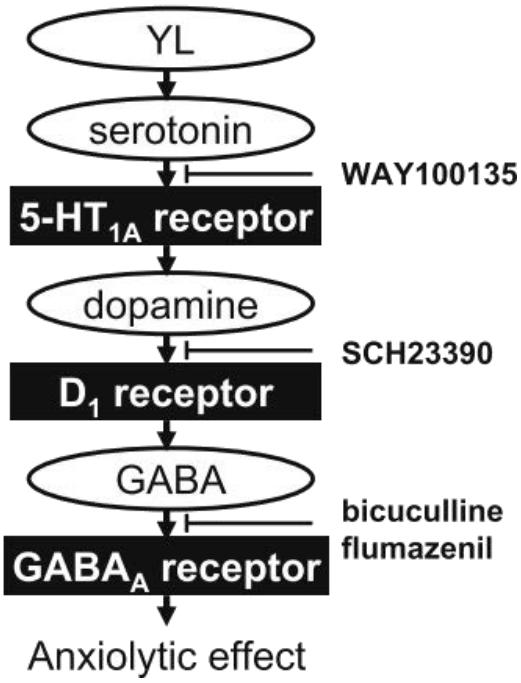


FIGURE 2.12 – A model of the central mode of action for YL anxiolytic-like activity.
(Kanegawa *et al*, 2010).

Now that the benzodiazepines and α-CZP have been introduced, through the prism of the GABAergic system and its implication in anxiety regulation, it would henceforth be interesting to better understand what anxiety is, and how it is regulated by the central nervous system.

3 BEHAVIOURAL AND NEURAL BASES OF ANXIETY

Contents

Anxiety is a complex phenomenon with several components, including social and psychological ones. This concept undertook a constant evolution during the past centuries, and this evolution may not even be complete yet. This division will review these different stages of the development of the concept of anxiety, starting with the psychological and behavioural angles (cf. ***3.1***). The neural circuits underlying this phenomenon will then be reviewed, adopting a temporal perspective, from the detection of a threat to the response initiation (cf. ***3.2***). Eventually, the disorders associated with high levels of anxiety, and their treatments, will be reviewed (cf. ***3.3***).

3.1 Anxiety: an adaptive behavioural response to threat

From a historical point of view, the term of **anxiety** appeared for the first time in 1844 with the Danish philosopher Søren Kierkegaard mentioning it in the title of his book *The Concept of Anxiety* (*Begrebet Angest*). This term was borrowed from Latin *angustus*, 'narrow, tight', itself derived from the verb *angere*, 'choke, squeeze'. Anxiety is then defined as the 'dizziness of freedom', felt by an individual when he is confronted to an infinity of possibilities. The German philosopher Martin Heidegger completed this first definition by adding the idea that 'anxiety is the fundamental mood of existence that places us in front of nothingness'. Anxiety is then formed from the liberty of choice in front of all possibilities, between the simultaneous existence of both attraction and repulsion. This is a first formulation of the conflict theory, which generates anxiety in the behavioural inhibition system (cf. ***3.2.1.3***).

Despite the fact that this term specifically appeared for the first time in the 19th century, different terms were previously used to describe what is known today as anxiety, with no precise consensus on the terms used (for a complete review, see (McReynolds, 1985)). As an example, John Locke, English philosopher of the 17th century, discussed about **uneasiness**: 'all pain of the body, of what sort so ever, and disquiet of the mind, is uneasiness'. This uneasiness leads to desire, and desire leads to action, which goals is to reduce the initial uneasiness (cf. ***3.1.1***). Causes of this uneasiness can be diverse, including physiological states (hunger, thirst, weariness, and tiredness) or passions (aversion, fear, and anger). This definition of Locke's uneasiness is close to modern definitions of anxiety.

It may also be noted the almost simultaneous apparition of English gothic novels at the end of the 18th century, where women shined in. For the first time, lectors were inclined to

experience apprehension without real causes through imaginative works. Some trace of these works can even be found in Voltaire correspondence: 'I don't believe it, but I'm still afraid of it'[3]. An historian signalled that before this period, people used to fear real dangers, such as famine, illness, or war. According to him, the apparition of fear without real cause, anxiety, matches with the disappearance of real fear with the augmentation of general security (Savelli, 2005).

The emergence of different psychiatric disorders conclusively gathered under the term of 'anxiety disorders' (cf. ***3.3.1***), finally led to the advent of anxiety with Sigmund Freud and his definitions of neuroses. According to him, anxiety could be distinguished into two different categories: objective or neurotic. Objective anxiety is the internal proportionate reaction to an external objective threat (i.e. a fire or a hurricane) is synonymous with fear. On the other hand, neurotic anxiety is a reaction to internal threats such as repressed sexual or aggressive impulses that threaten to enter consciousness. It arises specifically in response to unacceptable impulses (e.g. oedipal conflicts) which were punished in childhood and then repressed. According to Freud, anxiety is then expressed if one is re-experiencing these inner impulses, and fears the punishment, these reactions being disproportionate to the level of threat. A medical dimension of anxiety is for the first time stated, after being put aside by this discipline.

Indeed, in the 18th century, the first classification of fears in Lavoisier's *Medical dictionary* (1771) did not evoke anxiety, not more than the *Dictionary of medical sciences in 100 volumes* (*Dictionnaire des sciences médicales en 100 volumes*) published between 1864 and 1889 by Amédée Dechambre. Moreover, in French psychiatric literature, 'anxiety' is only mentioned three times in the 19th century and always as a reference to melancholia. It was only seized by the psychiatry discipline in the second half of the 20th century, where it became the main symptom of psychopathology (cf. ***3.3***).

The philosophical aspect of anxiety would still be carried on in the 20th century with authors like Jean-Paul Sartre, based on Kierkegaard works: 'it is in anxiety that humankind takes conscience of its liberty'[4]. Amongst existentialists, philosophical branch to which Sartre

[3] '*Je n'y crois pas, mais j'en ai peur*'
[4] '*C'est dans l'angoisse que l'Homme prend conscience de sa liberté'*

belongs, there is still this distinction between fear (against the external world, 'hell is other people'[5]) and anxiety (against oneself).

3.1.1 Anxiety in physiology: an emotional mechanism of defence

This section further defines anxiety through the eyes of psychiatrists and biologists. According to Oxford dictionary (*Oxford Dictionary of English*, 2010), anxiety is either the 'worry over the future or about something with an uncertain outcome', the 'uneasy concern about a person, situation, etc.' or 'a troubled state of mind arising from such worry or concern'. These definitions consider the different philosophical points defined above as Kierkegaard and Heidegger's notions of choice and conflict, Freud's reaction in a threatening situation, and Locke's state of mind triggered by these situations (cf. ***3.1***). The notion of anticipation is conserved by psychiatrists as anxiety is 'the anticipation of future threats' (American Psychiatric Association, 2013).

According to physiologists, anxiety is described as an adaptive response with three successive steps and components. Automatic physiological changes (increased heart rate, respiratory rhythm, piloerection, blushing…) are triggered by a threatening stimulus and regulated by neurotransmitters and hormones. They are followed by behavioural responses (jumping, freezing or aggression, facial and vocal expression), which eventually lead to a subjective experience. Charles Darwin initially studied these components in the late 1800s (cf. ***3.2***). Anxiety thus meets Kleinginna and Kleinginna criteria for their definition of emotion:

> an emotion is a complex set of interactions among subjective and objective factors, mediated by neural/hormonal systems, which can: (a) Give rise to affective experiences such as feelings of arousal, pleasure/displeasure; (b) Generate cognitive processes such as emotionally relevant perceptual effects, appraisals, labelling processes; (c) Activate widespread physiological adjustments to the arousing conditions; and (d) Lead to behaviour that is often, but not always, expressive, goal-directed, and adaptive (Kleinginna and Kleinginna, 1981)

[5] '*L'enfer, c'est les autres*'

As far as behavioural component is concerned and according to Gray (Gray, 1982), anxiety is expressed when the danger has not yet happened, when entering a threatening place or when the individual is going to an unfamiliar place, but where there is still a way to escape ('avoidable danger to approach', ***FIGURE 3.1***). As a survival mechanism of defence for the individual, preparing the organism to upcoming threats, anxiety has then survived through evolution. Neurological components of anxiety will be detailed later in this introduction (cf. ***3.2***).

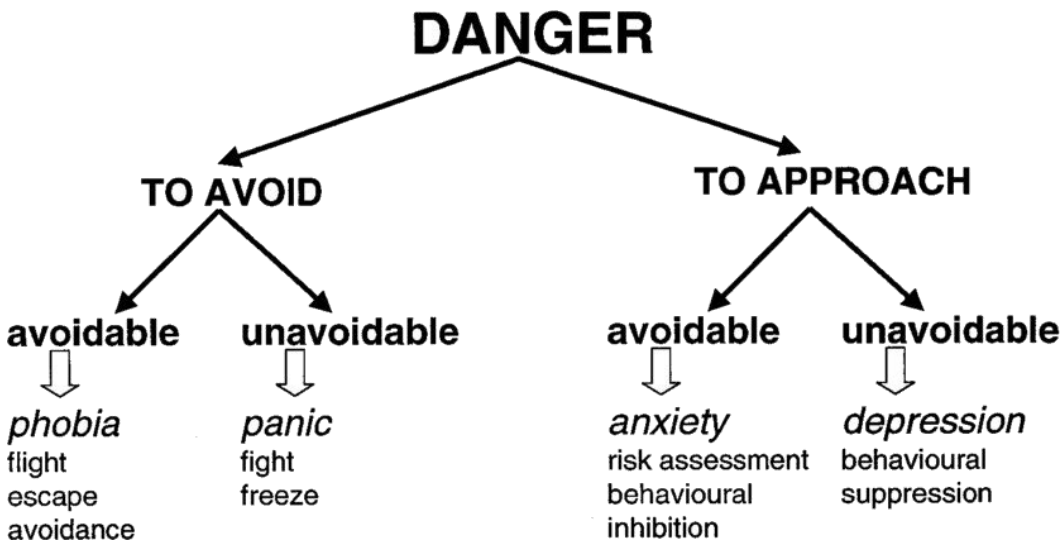


FIGURE 3.1 – Classification of responses and associated disorders in front of different classes of danger.
Diverse behaviours can be scored in the presence of different types of danger. These behaviours, observed in the animal reign, can be then associated with clinical disorders observed in humans (Gray, 1982).

3.1.2 Sources of confusion: stress and fear

The term of **stress** (term derived from old French *estrece*, narrowness, oppression) is often used when describing anxious states. This term is even more recently appeared than anxiety, the idea being taken from mechanisms where stress 'is the sum of the forces that neighbouring particles of a continuous material exert on each other, resulting in the deformation of the material (strain)'. The concept was then adapted to biology and popularised by Hans Selye, an Austro-Hungarian endocrinologist, who defined stress as 'an organism's response to a stressor such as an environmental condition' (Selye, 1950). The origin of this concept lies in the fact that non-specific, stereotyped answers caused by different external agents can be observed

amongst animals, these reactions being driven by the hyperactivity of the adrenal glands. The General Adaptation Syndrome (GAS) goal is to re-establish the threatened balance of the organism. GAS unfolds in three phases, defined as the alarm reaction, the resistance phase and finally the exhaustion phase. Selye, in the early 1900s, demonstrated for the first time that, unlike many other diseases, the organism's response was pathogenic but not the threatening agent. Anxiety is part of the psychological component of stress.

Anxiety is also often confused with **fear**. Compared to stress, the distinction between fear and anxiety is far vaguer. According to Oxford dictionary, fear is 'the emotion of pain or uneasiness caused by the sense of impending danger, or by the prospect of some possible evil' (*Oxford Dictionary of English*, 2010). The definition is opposed to that of the American Psychiatric Association, that distinguish fear and anxiety on a temporal matter, considering that 'fear is the emotional response to real or perceived imminent threat, whereas anxiety is anticipation of future threat' (American Psychiatric Association, 2013). This distinction was already mentioned before to discriminate fear and anxiety: 'anxiety has at least two defining characteristics: (i) it is an emotional state, somewhat resembling fear, and (ii) the disturbing stimulus which is principally responsible does not precede or accompany the state but is "anticipated" in the future' (Estes and Skinner, 1941). A distinction between the two terms has also been introduced by Blanchard and Blanchard, anxiety being felts while approaching a danger, fear being elicited during the escape (cf. ***FIGURE 3.1***) (Blanchard and Blanchard, 2003). Heidegger also distinguished fear and anxiety, considering that fear is about something particular whereas anxiety is more indeterminate. While philosophers and psychiatrists argue on differences between these two words, for a great number of biologists, anxiety and fear are parts of the same phenomenon. Indeed, both are adaptations of the organism, being the ability to sense threatening situations and being an evolutionary key to survival (Kim and Gorman, 2005). On a neurological point of view, anxiety even derives from fear neural mechanisms, involving the same cerebral regions (LeDoux, 2000, 2003) (cf. ***3.2***)

In both English and German, 'anxiety', 'dread', 'angst' or 'anguish' are used indistinctly to call the same phenomenon. In French, on the other hand, there is a distinction between 'anxiety' (*anxiété*) and 'angst' (*angoisse*), which definitions change between the authors. For some of them, anxiety is the psychological part of the phenomenon while angst embodies the somatic symptoms, whereas for other authors, it is the opposite. Some will consider angst as being an intense anxiety, other considering on the other hand angst as being acute, while anxiety is more

durable. Eventually, some will define angst as the philosophical aspect while anxiety would be the physiological facet of the same phenomenon. We remain on that definition, using the term 'anxiety' to describe both these phenomena in the following chapters.

3.1.3 State and trait anxiety

In the 1960, Spielberger developed a multifactorial anxiety theory, distinguishing anxiety as a reaction and anxiety as an underlying predisposition to respond to threats (Spielberger, 1966). The former was named state-anxiety, the latter trait-anxiety.

State-anxiety is a discrete transitory response to a given threatening stimulus in a given situation (Lister, 1990). It is often consciously perceived by the individual (Endler and Kocovski, 2001), and it involves an activation of the autonomic nervous system, triggering different physiological and behavioural changes: sweating, pounding heart, increased pulse rate and respiration; behavioural inhibition, vigilance… It is comparable to Freud's objective anxiety (cf. ***3.1***).

Trait-anxiety, on the other hand, does not vary over time, being an enduring characteristic of an individual, meeting Gordon Allport's trait theory (Lister, 1990). It however represents one's predisposition to experience anxious symptoms in specific situations, involving anxiogenic stimuli or not. Trait-anxiety amplifies state-anxiety as individuals diagnosed with high trait-anxiety levels are more prone to experience higher levels of state-anxiety compared to individuals with lower levels of trait-anxiety (Graziani, 2008). A comparison between kinetic energy (state-anxiety) and potential energy (trait-anxiety) could thus be made, e.g. a subject experiencing arachnophobia may have a normal trait-anxiety level leading to low state-anxiety levels under most circumstances, but when a spider is introduced, his state-anxiety will instantly jump (Endler and Kocovski, 2001). Trait-anxiety is close to Freud's definition of pathological neurotic anxiety (cf. ***3.1***).

3.2 Neural circuits of anxiety

The neural system is at the interface of an organism and its environment, integrating internal state and external stimuli in order to produce an appropriate behaviour. Emotions are a particular category of individuals' psychological experiments that played a role in survival. The theory started out with Charles Darwin in 1872 with his book *The Expression of the Emotions in Man and Animals* where he pointed out that emotions were developed as a result of natural selection and that they can then be observed across species.

The first central circuit of emotions was coined by James Papez in 1937 (***FIGURE 3.2***) (Papez, 1995). This circuit was described as a loop between the cerebral cortex, the thalamus, the hypothalamus and the hippocampus. The cortex and the hypothalamus were respectively responsible for the generation of feelings and bodily responses. Despite mentioning brain areas that are still described in processing emotions, this circuit was still incomplete.

The next proposition was then formulated in 1949 by Paul MacLean and his triune architecture theory (MacLean, 1949, 1952). According to him, the brain was a construction of three different entities assembling themselves as Russian dolls, the most inner one being the least evolved and conversely:

- **The reptilian complex**, composed of the brain stem was responsible for instinctual behaviours such as aggression, dominance or reproduction.
- **The paleomammalian complex or limbic system**, first described anatomically by Paul Broca in 1877 (Broca, 1877), was dedicated to the emotion generation. It was composed of all the structures of the Papez circuit, including new ones such as the amygdala and the prefrontal cortex.
- **The neomammalian complex** is composed of the neo cortex and process higher cognitive functions such as language, abstraction or planification.

This model however suffers from different critics. It has extensively been proven since then that the brain works as a whole and that complex tasks often needs the intervention of several brain regions, regardless of their triune complex situation. This postulate also works for emotions as it has been demonstrated that only two brain regions (the cingulate cortex and the amygdala) were always related to emotional states using fMRI (Phan *et al*, 2002). There is thus

not a specialised system in the treatment of emotions but instead several systems regulating the diversity of emotions (LeDoux, 2000; Millan, 2003).

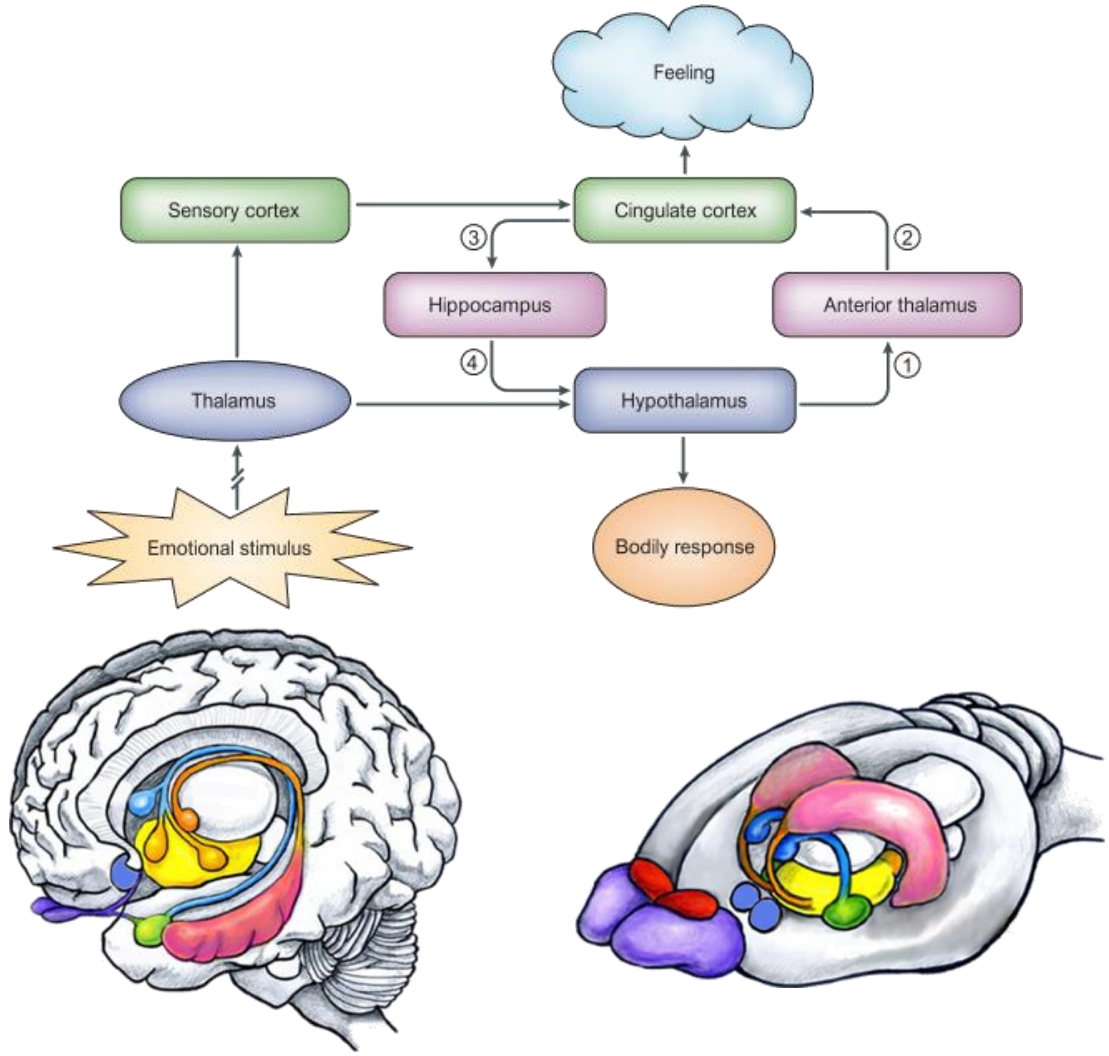


FIGURE 3.2 – The evolution of the emotional brain.

Top: The Papez circuit. Emotional stimuli are processed by the thalamus, which send outputs to both cortices and hypothalamus. Cingulate cortex produces 'feelings' while hypothalamus is responsible for bodily responses. Top-down and bottom-up loops are made via hippocampal formation and anterior thalamus (Dalgleish, 2004).

Bottom: Comparison of the limbic system in humans (left) and rodents (right). Accumbens nucleus (dark blue), amygdala (green), bed nucleus of the stria terminalis (BNST, light blue), hippocampal formation (pink), hypothalamus (yellow), olfactory bulbs (purple and red). Adapted from (Sokolowski and Corbin, 2012).

Several models have thus been proposed to model central circuits of anxiety. This section will adopt a temporal point of view using a model proposed by Calhoon and Tye (***FIGURE 3.3***) (Calhoon and Tye, 2015): once the threat has been detected by the organism via the sensory cortices and the thalamus (this step will not be reviewed here), a first set of regions will interpret the threat (cf. ***3.2.1***), coding the threat with an emotional value. The next step will be to evaluate the threat (cf. ***3.2.2***) and to take a decision about the most appropriate behaviour to adopt. Eventually, specific regions will triggers the physiological and motor components of the adopted behaviour (cf. ***3.2.3***).

Capital information is to keep in mind that these circuits are loops rather than one-way stream of information, making it harder to fully understand the flows between brain regions. Anxiety, as other complex phenomenon, do not rely on a single brain region, and is rather the result of an interaction between different regions (Stein and Steckler, 2010). Moreover, the difference between pre-clinical and clinical results may intensify the difficulty to fully understand this phenomenon.

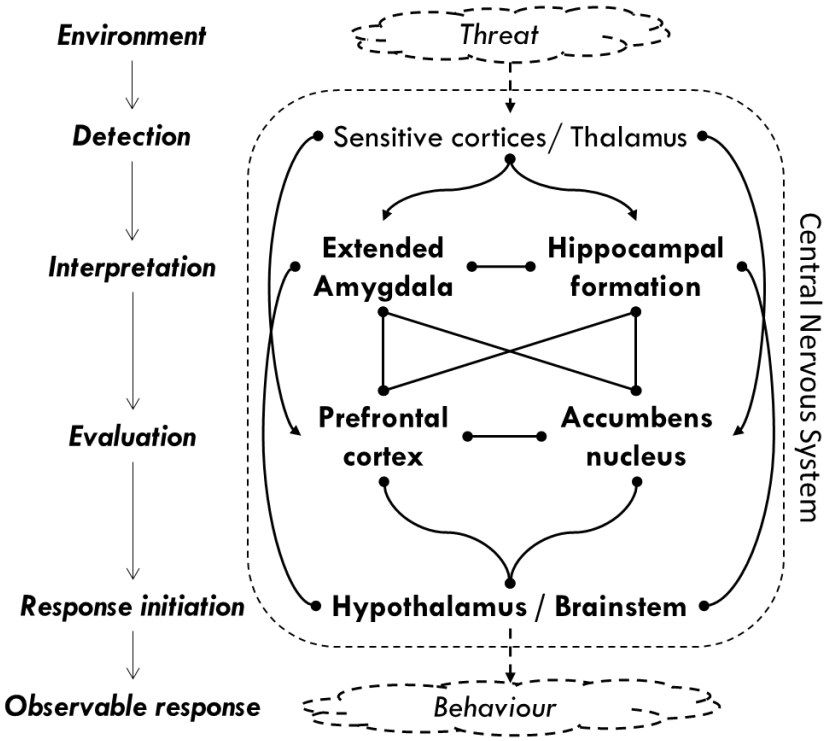


FIGURE 3.3 – Circuit organisation in anxiety.

Adapted from (Calhoon and Tye, 2015).

3.2.1 Interpreting the threat

3.2.1.1 Amygdala

Amygdala was not considered as a part of the limbic system at the beginning and was supposed to be a defensive structure at the first time, monitoring for instance aggression. Heinrich Klüver and Paul Bucy discovered in 1939 that removing temporal lobes (which contain the amygdala) in monkeys made them less fearful of humans. They observed the same phenomenon amongst humans, coining the Klüver-Bucy syndrome. Later, in 1956, Lawrence Weiskrantz discovered that the sole ablation of the amygdala led to the same observations. Since then amygdala has been extensively studied for fear and especially fear conditioning, but its role in anxiety is less evident due to the difficulty to specify the stimuli that triggers it (LeDoux, 2000; Steimer, 2002).

Concerning humans, it has been shown that a larger amygdala is correlated to anxiety and depressive disorders (Weniger *et al*, 2006), despite some studies showing that both enlarged and reduced amygdala volumes have been associated with anxiety disorders in humans (Tovote *et al*, 2015). It has also been shown that some anxiety disorders (post-traumatic stress and social anxiety) were associated with a increased activity of the amygdala using neuroimaging techniques (Rauch *et al*, 2003). Another study displayed the fact that amygdala was implicated in the process taking place while fleeing from a virtual predator (fMRI study) (Mobbs *et al*, 2007).

As far as rodents are concerned, the amygdala is an heterogeneous region composed of 13 distinct nuclei, distinguished on histological criteria such as the density, configuration, shape and size of stained cells, the trajectory of fibres, and/or the chemical signatures (***FIGURE 3.4***) (Pitkänen *et al*, 1997). Nomenclature of the nuclei will often differ amongst the authors.

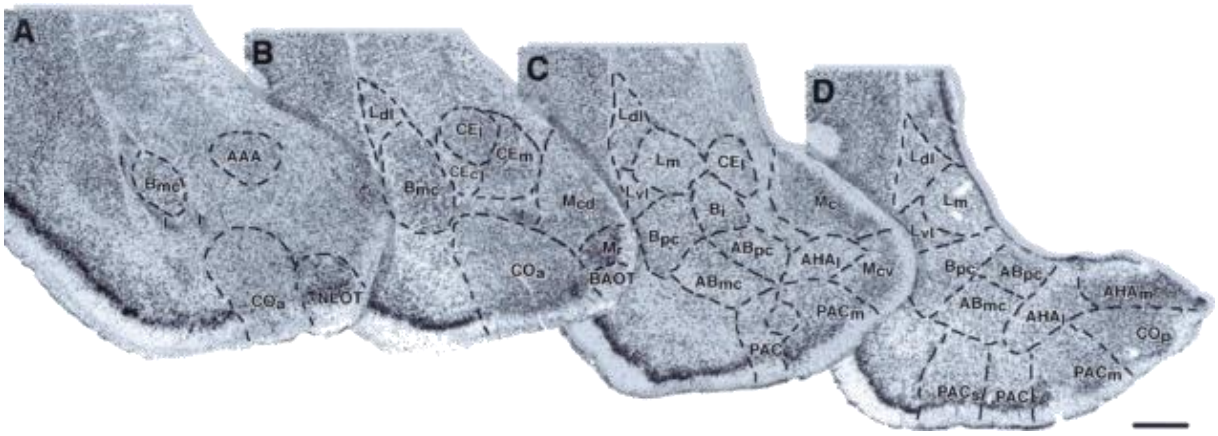


FIGURE 3.4 – Structural organisation of the amygdala.

Panel A is most rostral and panel D is most caudal. Scale bar 0.5 mm.

Deep nuclei

- Lateral nucleus (L): *dorsolateral division (L_{dl}), ventrolateral division (L_{vl}), medial division (L_m)*
- Basal nucleus (B): *magnocellular division (B_{mc}), intermediate division (B_i), parvicellular division (B_{pc})*
- Accessory basal nucleus (AB): *magnocellular division (AB_{mc}), parvicellular division (AB_{pc})*

Superficial nuclei

- Nucleus of the lateral olfactory tract (NLOT)
- Bed nucleus of the accessory olfactory tract (BAOT)
- Anterior cortical nucleus (CO_a)
- Medial nucleus (M): *rostral division (M_r), central division dorsal part (M_{cd}), central division ventral part (M_{cv}), caudal division (M_c)*
- Periamygdaloid cortex: *periamygdaloid cortex (PAC), periamygdaloid cortex, medial division (PAC_m), periamygdaloid cortex, sulcal division (PAC_s)*
- Posterior cortical nucleus (CO_p)

Other amygdaloid areas

- Anterior amygdaloid area (AAA)
- Central nucleus (CE) : capsular division (CE_c), lateral division (CE_l), intermediate division (CE_i), medial division (CE_m)
- Amygdalo–hippocampal area (AHA): medial division (AHA_m), lateral division (AHA_l)
- Intercalated nuclei (I)

(Pitkänen *et al*, 1997)

The basolateral nucleus (BLA) is often considered as the gatekeeper of the amygdala, receiving the principal outputs of the structure, coming from thalamus and sensory cortices (***FIGURE 3.5***) (LeDoux, 2003, 2007; LeDoux *et al*, 1990). Other nuclei also collect sensory information such as the medial nucleus (MeA) with olfactory cues from predators and the basomedial (BMA) with auditory and visual cues (Gross and Canteras, 2012). Taken together this suggests that the amygdala is a crucial relay for sensory information in the brain and it occupies a central role in integrating the sensory clues related to the threat to then trigger appropriate behavioural cues (Stein and Steckler, 2010). However the amygdala receives also information coming from other structures such as the prefrontal cortex or the hippocampal formation (LeDoux, 2003).

Information mostly travels unidirectionally from the BLA to the central nucleus (CeA) which is the main output structure of the amygdala (***FIGURE 3.5***) (Gilpin *et al*, 2014). A major part of the information will flow to the hypothalamus and the brainstem where adapted behaviour will be initiated (***FIGURE 3.6***, cf. ***3.2.3***).

Nuclei of the amygdala do not mediate behaviours the same way: a complete inactivation of the amygdala is anxiolytic in the elevated plus-maze but only the CeA (and not the BLA) is needed for the avoidance of open spaces, which is an anxiogenic component of this device (Moreira *et al*, 2007). In a more subtle approach, the pharmacological activation of the BLA in rats triggers anxiety but the activation of the specific fibers connecting the BLA to the CeA decreases anxiety (Tye *et al*, 2011). As seen with the clinical results above, classifying the amygdala as anxiolytic or anxiogenic is then oversimplifying (Adhikari, 2014).

Concerning anxiety-related drugs, using a marker of neuronal activity (cf. ***4.2***), it was demonstrated that different anxiogenic drugs increased the neuronal activity of the amygdala in rats (Singewald *et al*, 2003) despite some other authors reporting that this neuronal activation in the CeA can be found regardless of the anxiogenic or anxiolytic property of the drug used (Thompson and Rosen, 2006). The CeA is also the main mediator of the anxiolytic properties of benzodiazepines in rats (Carvalho *et al*, 2012).

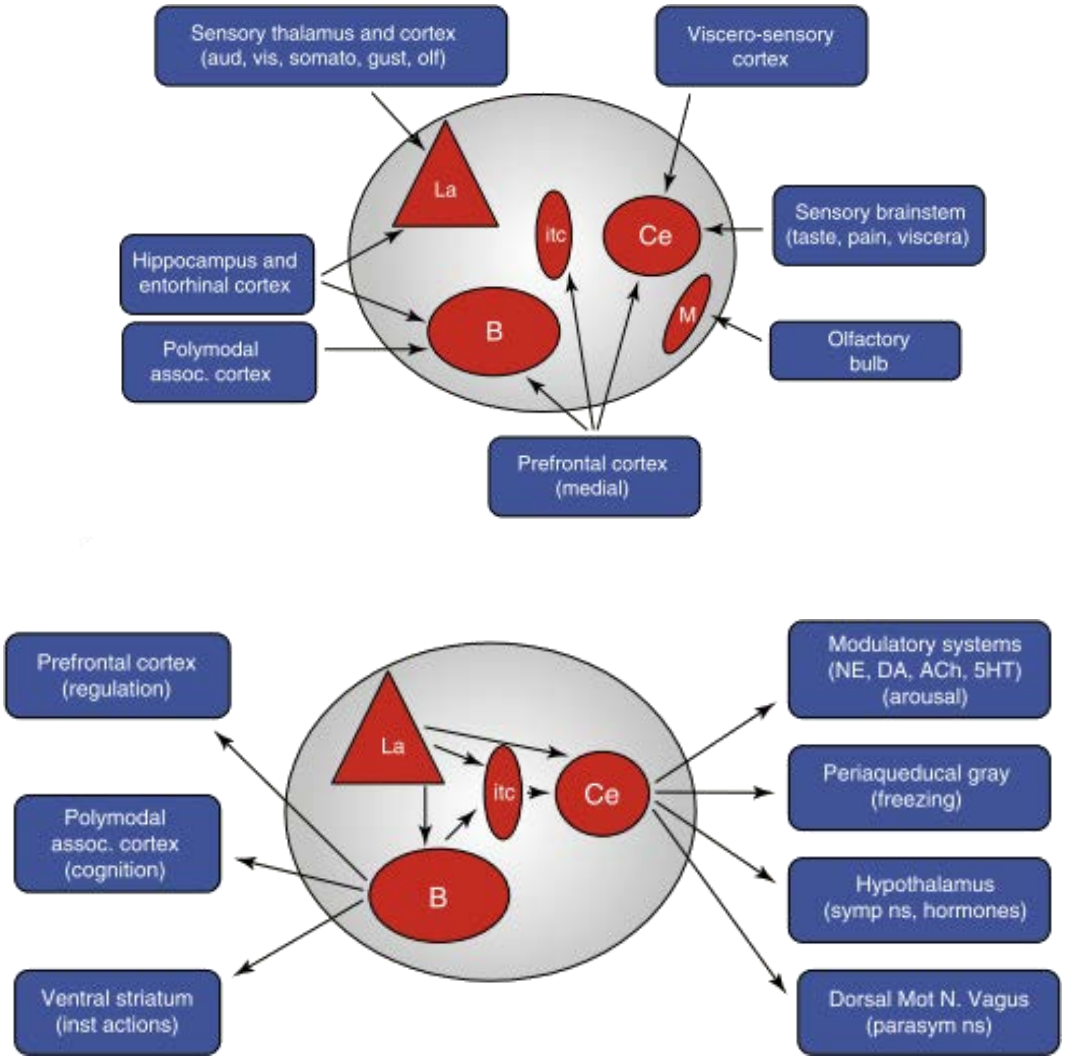


FIGURE 3.5 – Inputs and outputs of the amygdala.

Abbreviations of amygdala areas: B, basal nucleus; Ce, central nucleus; itc, intercalated cells; La, lateral nucleus; M, medial nucleus. Sensory abbreviations: aud, auditory; vis, visual; somato, somatosensory; gus, gustatory (taste). Modulatory arousal system abbreviations: NE, norepinephrine; DA, dopamine; ACh, acetylcholine; 5HT, serotonin). Other abbreviations: parasym ns, parasympathetic nervous system; symp ns, sympathetic nervous system (LeDoux, 2007).

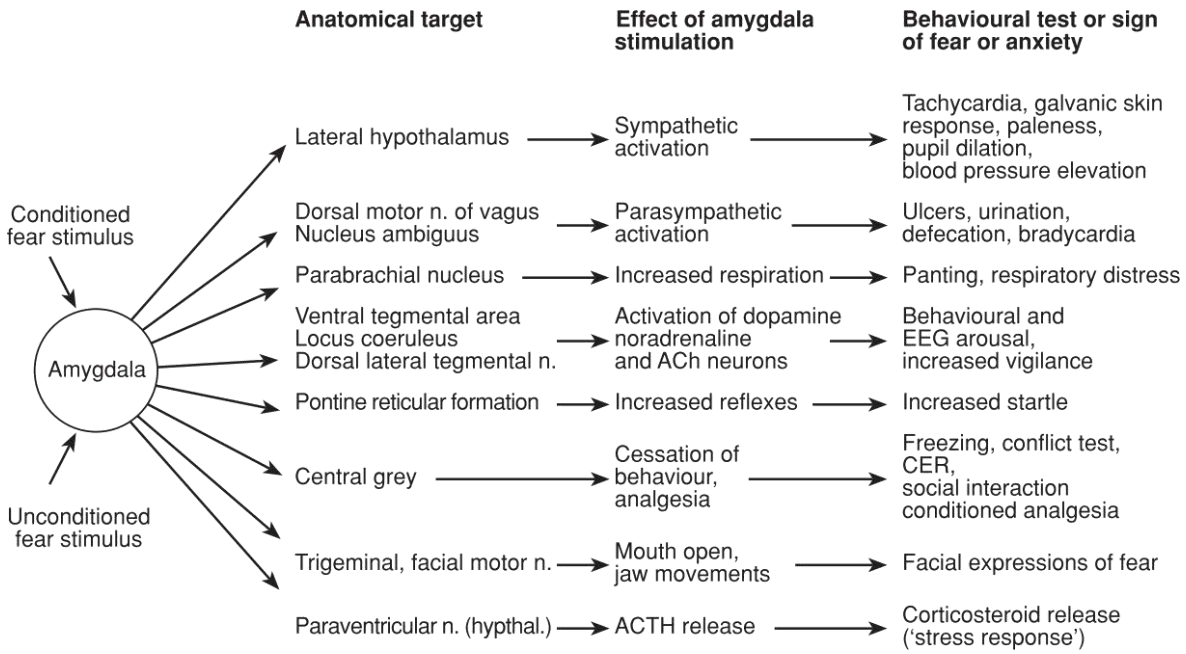


FIGURE 3.6 – Connections of the central amygdala nucleus.

Schematic diagram showing direct connection between the central nucleus of the amygdala and a variety of hypothalamic and brainstem target areas that may be involved in different animal tests of fear and anxiety. n, nucleus; hypothal., hypothalamus; ACh, acetylcholine; ACTH, adrenocorticotrophic hormone; EEG, electroencephalogram; CER, conditioned emotional response (Davis *et al*, 1994).

Eventually, some nuclei display high densities of GABAergic neurons (Bian, 2013; Capogna, 2014) but most of them are gathered as intercalated cells refining amygdala information flows as inhibitory interneurons (Lee *et al*, 2013). $GABA_A$ receptors display a specific profile depending on the nuclei, the central amygdala being more concentrated with receptors mediating the anxiolytic properties of benzodiazepines (cf. ***2.2.3***) (Fujimura *et al*, 2005; Kaufmann *et al*, 2003). Opioids receptors are also found in this region, also mediating the anxiolytic effects of benzodiazepines (Primeaux *et al*, 2006; Randall-Thompson *et al*, 2010). Conversely, it has also been shown that some benzodiazepines (midazolam, chlordiazepoxide, and diazepam) had an affinity for both κ and δ receptors, but not for μ receptors (Cox and Collins, 2001).

To sum up the role of the amygdala in anxiety (***FIGURE 3.7***), by receiving quick information of sensory cues via the thalamus, amygdala can trigger fast appropriate behaviour by associating the sensory cues with an emotional value (e.g. the view of a cat as threatening). However, this first information is rather sketchy and the intervention of other areas is needed to precise the sensory cues. Reinterpretation is also sometimes needed, as the first emotional values associated by the amygdala may be wrong (e.g. associating the view of a hose with a snake).

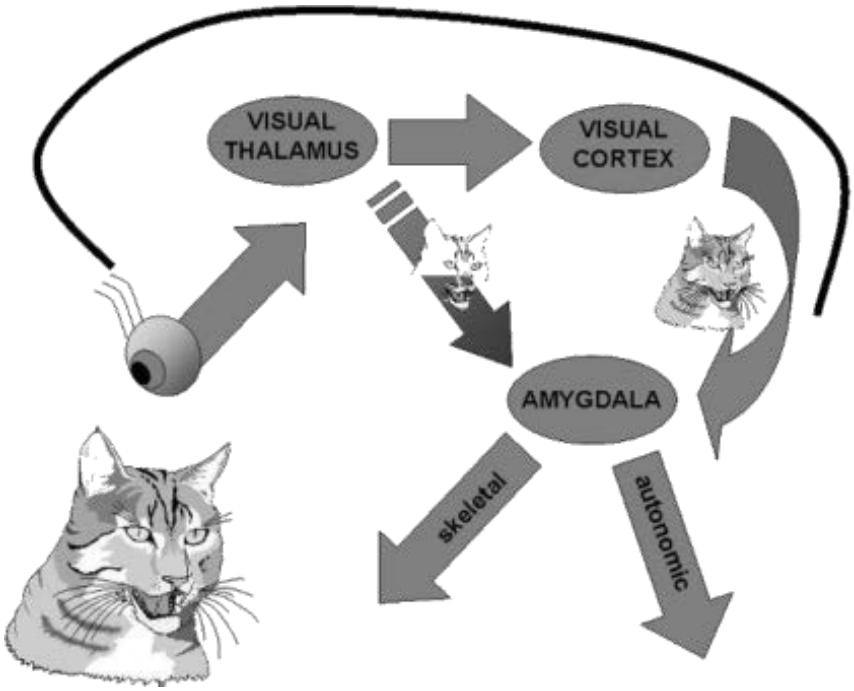


FIGURE 3.7 – 'Quick and dirty' versus 'slow and sophisticated' interpretation.

The cat produces a visual stimulus which will be received by the retina and processed by the visual thalamus. The thalamus then sends a sketchy information ('quick and dirty' interpretation) to the amygdala which allows an escape response in the minimum of delay. A longer way ('slow and sophisticated' interpretation) goes through the visual cortex for a more detailed classification of the stimulus, and then also reaches the amygdala. This second information either completes the escape response, or cancels it if the 'quick and dirty' interpretation triggered a false alarm (Gray, 1982).

3.2.1.2 Extended amygdala: the Bed Nucleus of the Stria Terminalis (BNST)

Some studies pointed out the fact that lesions in the amygdala do not automatically affect behavioural tests as the elevated plus-maze or conditioned defensive burying while diazepam still exerted it anxiolytic effects in a model of amygdala lesioned animals (Steimer, 2002). Other brain structures must then be also involved in the regulation of anxiety, relaying information that normally passes through amygdala. The **bed nucleus of the stria terminalis (BNST)** is one of them. BNST is similar to the amygdala in terms of neuropeptide expression and morphology and has thus be integrated to a complex called the extended amygdala (Adhikari, 2014). It also receives strong projections from the amygdala itself as well as the hypothalamus and the brainstem.

Patients with a generalised anxiety disorder displayed an increased activity of the BNST when put in a gambling situation with high uncertainty, eliciting a sustained anxiety feeling (Yassa *et al*, 2012). As for the amygdala, experiments with rodents displayed conflicting results because of the existence of subnuclei within the BNST. Activation of specific subnuclei do not trigger the same behavioural cues (Adhikari, 2014; Calhoon and Tye, 2015).

BNST is then thought to mediate sustained fear/anxiety stimuli while amygdala will process more acute or phasic responses (Walker *et al*, 2003). As an example, the BNST can act as a compensatory area to treat anxiety in BLA-lesioned rats, but the animals need more training to fully compensate the lesioned region (Poulos *et al*, 2010). Simplifying, BNST is the key to trait-anxiety while amygdala mediates state-anxiety.

3.2.1.3 Hippocampal formation

The **hippocampal formation** has been extensively described for its role in memory, spatial learning and navigation. However, this region can also work as a context analyser in anxiety regulation. Anatomically speaking, the dorsal hippocampal formation is responsible for spatial memory while the ventral part of it mediates anxiety (Bannerman *et al*, 2014). This was based on the fact that permanent or temporary lesions of the hippocampal formation acted the same way as anxiolytic drugs on behavioural tests.

Another reason is that the hippocampal formation is the main actor of the 'Behavioural Inhibition System' (BIS, ***FIGURE 3.8***) described by Gray (Gray, 1982). When put in an anxiogenic situation, i.e. a situation of conflict or uncertainty (e.g. signals of punishment, signals of non-reward, novel stimuli and innate fear stimuli, cf. ***3.1***), the organism, driven by the central nervous system, has to adopt an appropriate behaviour to resolve that conflict. This results in increased arousal, increased attention and the suppression of ongoing moto programmes (behavioural inhibition). The role of the BIS is then to compare actual and expected stimuli: if there is a difference between them, the BIS is activated and the behaviours are inhibited, while attention and arousal are increased. Anxiolytic drugs can affect this system, by decreasing the neural activity of the hippocampal formation for instance, lowering the organism physiological responses (de Medeiros *et al*, 2005).

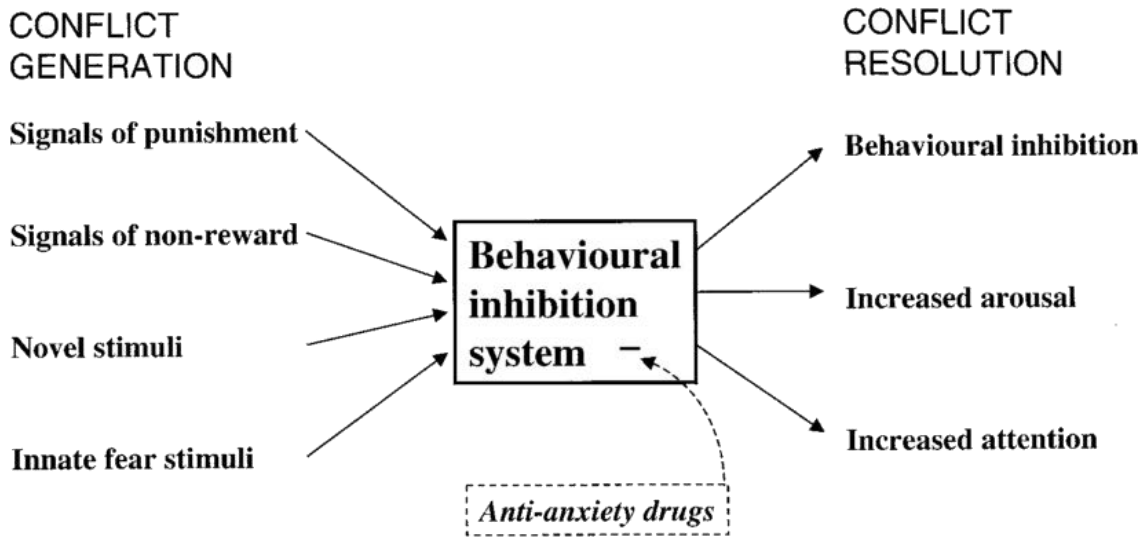


FIGURE 3.8 – The behavioural inhibition system theory.

The behavioural inhibition system is triggered in the case of an avoidable danger to approach. The individual responds to any of the inputs (conflict generation) with all of its possible outputs (conflict resolution) (Gray, 1982).

This interpretation role of sensory cues by the hippocampal formation can be illustrated with animal experiments. For example, rats can be habituated to two separate audio-visual cues (e.g. a tone followed by a constant light versus a click followed by a flashing light). When auditory and visual cues are mismatched a new behaviour will be developed by the animals to re-associate the two cues. However, rats with hippocampal lesions are not able to adapt themselves to the new cues, displaying a inconsistency to interpret sensory cues (Honey *et al*, 1998).

Concerning clinical results, it has also been observed that the blood-oxygen-level dependent (BOLD) signal in the hippocampal formation was reduced within anxious volunteers in an anxiogenic situation triggered with visual cues compared with a control situation (Wik *et al*, 1993).

3.2.2 Evaluating the threat

3.2.2.1 Prefrontal cortex

Anxiety arises from activity when ambiguous stimuli are evaluated as threatening (Calhoon and Tye, 2015). For instance, a loud noise can be interpreted differently by two individuals resulting in opposing behaviours: one can think of a firework and start to look after it, while another may think about a gunshot and start to hide and protect. This is where the prefrontal cortex (PFC) is thought to act in anxiety regulation.

In a general sense, the PFC is the logic and cognitive modulation centre of anxiety behaviours, opposing the amygdala, which is the emotional centre. The two regions interact with each other, the PFC regulating amygdala outputs, despite the fact that this interaction is not yet fully understood (Cardinal *et al*, 2003; Kim and Gorman, 2005). Just as above described regions, the PFC is composed of different regions that respond differentially to different situations (e.g. decision making) (Sul *et al*, 2010).

The same has been observed amongst humans during fMRI studies. A reduced BOLD signal in the PFC is observed in anxious patients during anxiogenic visual cues (Wik *et al*, 1993) or amongst women suffering from post-traumatic stress (Bremner *et al*, 1999) while an increased activity was noted in the same region with a remote virtual predator (Mobbs *et al*, 2007).

An increase of neuronal activity markers (cf. ***4.2***) in the PFC has been observed in rodents in different anxiogenic situations (Kovács, 1998) or after the administration of anxiogenic drugs (Singewald *et al*, 2003). On the other hand, a decrease of the activity in the PFC was observed in genetically selected hyperanxious rats (Kalisch *et al*, 2004). Moreover connections between the BLA and the PFC have been shown to mediate the choice response in fear conditioning, underlining the role of this structure in threat evaluation (Cardinal *et al*, 2003).

The greatest problem in studying the role of the PFC in anxiety regulation is the controversy regarding the equivalence of this regions across different species (Uylings *et al*, 2003). Indeed as anxiety is defined in humans as the capacity to worry about the future (cf. ***3.1***), this feature being mostly linked to a greater development of the PFC in primates, it is then hard to mimic it in behavioural tests using rodents. This specific brain region may then have a more prominent role in the regulation in human anxiety, that cannot be fully studied amongst rodents (Berkowitz *et al*, 2007).

3.2.2.2 Accumbens nucleus

Motivational effects of emotionally significant stimuli are mediated in part by the accumbens nucleus (Acb), which is part of the ventral striatum structure. As Acb is anatomically part of the limbic system, but receives strong dopaminergic projections from the brainstem, it has often been described as a 'limbic-motor interface' (Cardinal *et al*, 2003). This regions has been extensively studied for its role in motivation and reward, as well as aversion (Carlezon and Thomas, 2009; Ikemoto and Panksepp, 1999).

The drive state of an individual will then also have an impact on his behaviour (Calhoon and Tye, 2015). Indeed, in rodents, most tests are based on an approach-avoidance conflict (cf. ***4.1.1***), where the animal has to choose between a potentially dangerous but also potentially rewarding option (approach) or to stay in a safer option (avoidance). These types of behaviours can also be observed in the wild (e.g. going to the threatening water hole depending on the thirst level of the animal) and are partly driven by the Acb. Eventually, it has been proven that behavioural models have an influence on the plasticity of connections within the reward system (Carvalho *et al*, 2005). The role of this structure has also been studied in the context of fear conditioning (Cardinal *et al*, 2003).

3.2.3 Response initiation

3.2.3.1 Hypothalamic-pituitary-adrenal axis

The hypothalamus drives a certain number of critical behavioural functions of the organism and the survival of the species (e.g. eating behaviour, reproduction, circadian rhythms...) and fear/anxiety responses belong to them. In this way, clinical trials have spotted an activation of

the hypothalamus for patients with panic disorders (Kalueff and Nutt, 2007). The evaluation of neuronal activity using immunohistochemical markers (cf. ***4.2***) also spotted an increase of the hypothalamic activity amongst rodents during restraint (de Medeiros *et al*, 2005) or social stress (Lkhagvasuren *et al*, 2014).

The hypothalamic-pituitary-adrenal (HPA) axis plays a central role in the stress response of the organism, anxiety being a component of this response (cf. ***3.1.2***). This axis is activated after a stressful stimulus, resulting into the secretion of corticotropin releasing hormone (CRH) by a specific nucleus of the hypothalamus, the paraventricularnucleus (PVN) via the median eminence, a circumventricular (i.e. without blood brain barrier) organ (***FIGURE 3.9***). This triggers the release of adrenocorticotropin hormone (ACTH) and arginine-vasopressin hormone (AVP) by the pituitary gland, which targets the adrenal gland liberating glucocorticoids (cortisol for humans, corticosterone for rodents). Glucocorticoids have numerous physiological effects depending on their target organ: increase of cardiovascular and respiratory rhythms, activation of the sympathetic system, decrease of immunity response… Negative feedback loops are then mediated by the glucocorticoids to decrease the stress response of the organism.

The PVN receives different afferents projections coming from the limbic system coming from amygdala, BNST, hippocampal formation, prefrontal cortex and brainstem (***FIGURE 3.10***) (Herman *et al*, 2005). An over exposition of the brain to chronic stress (and thus chronic anxiety) with glucocorticoids has been proven to damage the brain, especially the hippocampal formation and the prefrontal cortex (Mah *et al*, 2016).

3.2.3.2 Brainstem

At last, the brainstem is also involved in the behavioural responses triggered by an anxiogenic situation, especially motor and autonomic responses.

The periaqueductal grey area (PAG) is one of the most studied brain regions in terms of anxious behaviours and it is supposed to mediate the fight/flight phenomenon of anxious behaviours. The freezing behaviour of animals in an anxiogenic situation has been linked to an increased activity of the PAG in rats (Borelli *et al*, 2005), while the presence of a close virtual predator also raises the activity of humans in an fMRI paradigm (Mobbs *et al*, 2007).

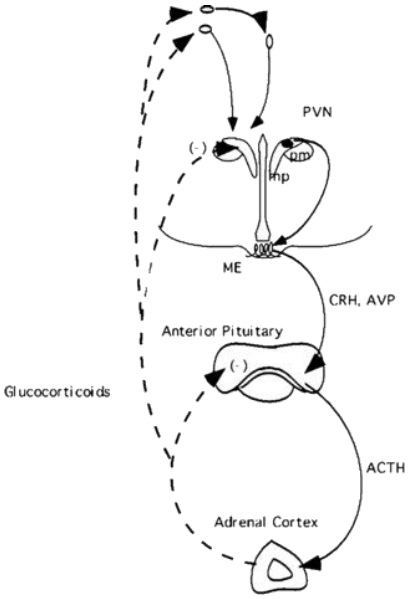


FIGURE 3.9 – The hypothalamic-pituitary-adrenal axis.

From head to bottom: PVN, paraventricular nucleus of the hypothalamus; ME, median eminence; CRH, corticotropin-releasing hormone; AVP, arginine-vasopressin hormone; ACTH, adrenocorticotropin hormone (Ziegler and Herman, 2002).

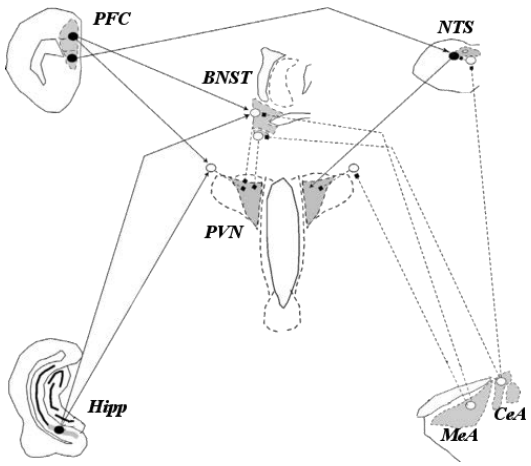


FIGURE 3.10 – Limbic afferent projections of the paraventricular nucleus of the hypothalamus.

BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; Hipp: hippocampal formation; MeA, medial nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus; NTS, nucleus of the tractus solitarius. Adapted from (Herman *et al*, 2005).

3.3 Pathology of anxiety

As seen above (cf. ***3.1***), anxiety is a survival mechanism to preserve an organism from threatening situations (state-anxiety). However, when the levels of anxiety are excessive or frequent, or when the individuals have a predisposition to experience high anxiety levels in non-threatening situations (high levels of trait-anxiety), this protective feeling can become a burden for the individual by leading to anxiety disorders (Tovote *et al*, 2015).

Each historical period uses specific medical key-words. As an example, the 19th century was haunted by fatigue, as a recollection of tuberculosis. The 20th century, and particularly its second half is known as the Age of Anxiety with the work of poets (W.H. Auden), authors (A. Camus), musicians (L. Bernstein), or philosophers (J.P. Sartre) (cf. ***3.1***) (Endler and Kocovski, 2001).

From the medical side, concern about anxiety disorders started at the end of the 18th century. Carl Westphal firstly described agoraphobia in 1867, even if it was at the time regarded as an ocular disorder. Jacob Mendes Da Costa, an American military physician, identified the 'irritable heart' amongst worn out soldiers in 1871, without mentioning fear nor anguish. It is now well known as an anxiety disorder symptom (Da Costa's syndrome). Albert Pitres and Emmanuel Régis defined 'erythrophobia', the fear of blushing, in 1897. Other examples can be cited, but the real recognition of anxiety disorders is attributed to Sigmund Freud in 1895 with his neurotic anxiety compared to objective anxiety (cf. ***3.1***). Neurotic anxiety was then described as urges (mostly sexual) that were punished during childhood leading to free-floating anxiety in adulthood if the urges are being expressed by fear of punishment. The anxiety reactions were disproportionate to the level of threat, which is also nowadays considered as the definition of anxiety disorders.

3.3.1 Anxiety disorders

Different disorders, defined by precise criteria, are categorised in the anxiety disorders section of the *Diagnostic and Statistical Manual of Mental Disorders, 5th edition* (DSM-V) (American Psychiatric Association, 2013). These disorders share the feature of disturbances associated with excessive fear and/or anxiety.

Separation anxiety concerns individuals being anxious towards the separation of an attachment figure to a degree being inappropriate to the development of the individual. This can be triggered by the anxiety of attachment figures' death, separation from attachment figures or the reluctance to leave attachment figures. While the symptoms often develop during childhood, they may persist during adulthood.

Selective mutism is a total inability for one to express himself in social situations where there is an expectation to speak (e.g. school, work), while the same individuals can speak in other situations.

Specific phobia is characterised as objects or situations triggering fear, anxiety or avoidance. These behaviours are usually immediately induced by the phobic situation, in a persistent and out-of-proportion fashion.

Social anxiety concerns individuals who are anxious in social situations or interactions where they have the possibility to be scrutinised. These include meeting unfamiliar people, situation where the individuals may be observed eating or drinking and situations where the individuals have to express themselves in front of others. The anxiety comes from the possible negative evaluation of being embarrassed, humiliated or rejected by others, or even by offending others.

Panic disorders relates to individuals experiencing recurrent and unexpected panic attacks. Individuals are constantly concerned and worried about having new attacks and thus change their behaviours in a non-adapted fashion to avoid these attacks (e.g. avoidance of unfamiliar places). Panic attacks are surges of intense fear or intense discomfort, going along with physical and/or mental symptoms, which peaks can be attained in a few minutes. They may be either *expected*, as in the case of a specifically feared object or situation, or *unexpected*, when they are triggered without apparent reason. Panic attacks are used to diagnose several disorders which are not limited to anxiety disorders (e.g. substance use, depressive and psychotic disorders).

Individuals suffering from **agoraphobia** are anxious in two or more of the following situations: using public transportation; being in open spaces; being in enclosed spaces; standing in line or being in a crowd; being alone outside of home in other situations. Individuals avoid

these situations, by being anxious of not being able to escape, or receive help in case of a panic attack or another incapacitating or embarrassing syndrome.

Individuals with **generalised anxiety disorders** (GAD) are excessively anxious and worried in several situations, including school or work, and the individuals cannot control these feelings. Furthermore, individuals experience physical symptoms such as restlessness or edginess; weariness; difficulty to focus; irritability; muscle tension; sleep disorders.

Eventually, the **substance/medication-induced anxiety disorders** include anxiety triggered by a substance intoxication or withdrawal of by a medication treatment.

In the previous edition of the DSM (American Psychiatric Association, 2000), **obsessive-compulsive disorders** (OCDs) were also included in the anxiety disorders. They nowadays have their own section entitled OCDs and related disorders. Nevertheless, many relations still exist between anxiety disorders and OCDs. This new section gathers all the disorders characterised by the presence of 'obsessions' (recurrent and persistent thoughts, urgent, or images that are experienced as intrusive and undesired) and/or 'compulsions' (repetitive behaviours or mental acts that an individual feels driven to achieve following an obsession or some specific rules that must be strictly applied). These disorders thus include:

- **OCDs**, properly speaking;
- **Body dysmorphic disorder**, which is characterised by an excessive preoccupation of one or more physical defects or flaws that are imperceptible to the others and is expressed by repetitive behaviours (e.g. mirror checking, excessive grooming, or constant reassurance seeking) or mental acts (e.g. comparing one's appearance to that of other people);
- **Hoarding disorder**, characterised by the difficulty to discard possessions, not matter what their actual value is, associated with both the need to save items and the distress to throw them away;
- **Trichotillomania** (hair-pulling disorder) is the recurrent pulling out of one's hair finally leading to alopecia;
- **Excoriation** (skin-picking disorder) is the constant picking of one's skin eventually causing skin lesions;
- **Substance/medication-induced OCDs**.

3.3.2 Prevalence of anxiety disorders and treatments

Most of the disorders cited above develop during childhood and persist if they are not treated. In 2007, the French general population's 12-month prevalence of anxiety disorders in France was estimated to be 15% while the lifetime prevalence is 21.6% (Haute Autorité de Santé, 2007; Leray *et al*, 2011). Considering each specific anxiety disorders, their 12-month and lifetime prevalences were respectively: GAD, 2.1% and 6%; panic disorder, 1.2% and 3%; agoraphobia, 0.6% and 1.8%; social phobia, 1.7% and 4.7%; specific phobia, 4.7% and 11.6%. Overall, the prevalence evaluated amongst women was twice as much of that evaluated amongst men (***TABLE 3.1***). These disorders have been shown to have high consequences on work efficiency, even often leading to job quit (Cohidon, 2007). Other comorbidities or psychiatric disorders were highly associated with anxiety disorders such as alcohol abuse (2.3%), drug addiction (1.8%) or depression (11.0%) (Cohidon, 2007; Hofmeijer-Sevink *et al*, 2012; Leray *et al*, 2011).

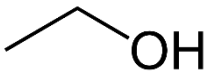
TABLE 3.1 – Prevalence of anxiety disorders in French population according to the age.
Adapted from (Cohidon, 2007).

	Men						Women					
		GAD	Panic	Agora-phobia	Social phobia	At least 1 AD		GAD	Panic	Agora-phobia	Social phobia	At least 1 AD
	n	%	%	%	%	%	*n*	%	%	%	%	%
Age												
18-30	4,810	12.9	4.2	1.7	4.8	22.1	5,136	15.1	7.4	2.6	7.5	29.9
30-40	3,659	12.1	3.4	1.5	3.5	19.1	3,977	16.1	5.4	2.7	5.0	26.8
40-50	3,260	12.3	3.4	1.1	2.9	19.0	3,650	16.0	5.4	3.4	5.8	27.8
50-65	4,005	9.7	2.7	1.7	2.7	15.8	4,480	15.1	5.4	3.2	5.6	26.5
65 and more	2,546	6.0	1.4	1.2	1.6	10.0	4,094	11.3	2.6	1.8	0.5	17.7
Total	18,820	10.7	3.1	1.5	3.2	17.4	21,337	14.5	5.1	2.7	5.2	25.4

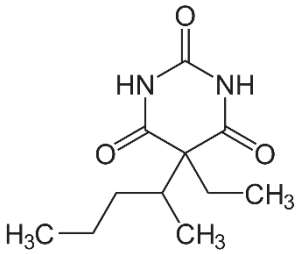
Anxiolytics have a clinical definition. They should reduce anxiety as defined in the anxiety disorders, described for instance by the DSM-V (cf. ***3.3.1***), without affecting any other psychological state or syndrome (Gray, 1982). Taken as a group, most drugs do correspond to the definition, as for instance they are inefficient to deal with schizophrenia or aggression (Gordon, 1975). However, taken alone, no drug has been shown to be specific of a specific anxiety disorder. Despite most drugs being able to deal with GAD, the problem is particularly flagrant with phobia, which do not have the same ethological and neurological basis than other anxiety disorders (Fredrikson *et al*, 1995). Some anxiolytics have also been used to deal with depression as these two disorders display high comorbidities.

Five generations of anxiolytics were introduced along time. The oldest anxiolytic drug was alcohol since ethanol potentiates the inhibitory effect of neurotransmitter GABA in the brain (Soldo *et al*, 1994) (cf. ***2.1.4***), but its numerous side effects (depressant, toxicity, addiction…) made it more than clinically unattractive. The second anxiolytics introduced were the barbiturates, which again increased GABA inhibitory effects in the brain (cf. ***2.1.4***). It is mostly used today as a sedative only. In the 1950s, thalidomide was introduced but immediately withdrawn from the market due to its teratogenic side effects. Meprobamate followed the same fate in 2012, being as unappealing as alcohol. The fourth generation, the BZD family or 'classical' anxiolytics, introduced in the 1960s, is still the most prescribed anxiolytic drugs today (cf. ***2.2***). Nevertheless, these drugs are now being challenged by a newer generation of anxiolytics, composed of buspirone and of a new use of antidepressant drugs.

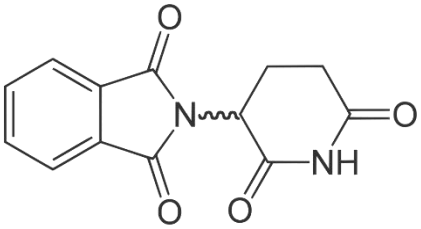
As far as BZDs are concerned, in 2012, 7 millions of French consumed at least one anxiolytic BZD (out of the 11.5 millions who consumed at least one BZDs, whatever the therapeutic effect is, over the year) (ANSM, 2013). Most consumers (66.6%) are women (***TABLE 3.2***) and a third of women over 65 consume at least one anxiolytic BZD daily. Despite a small decrease in BZD consumption (131 million BZDs' packages sold in 2012 against 134 million in 2010, the overall number of consumers remaining stable since 2007), France remains the second consuming country in Europe, behind Portugal. General practitioners prescribe 80% of the BZDs and the consumption time is higher than the recommended time (5 months against 12 weeks). Overconsumption is also mostly due to misdiagnosis (Pélissolo *et al*, 2007).



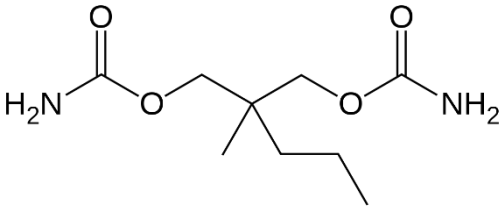
Ethanol



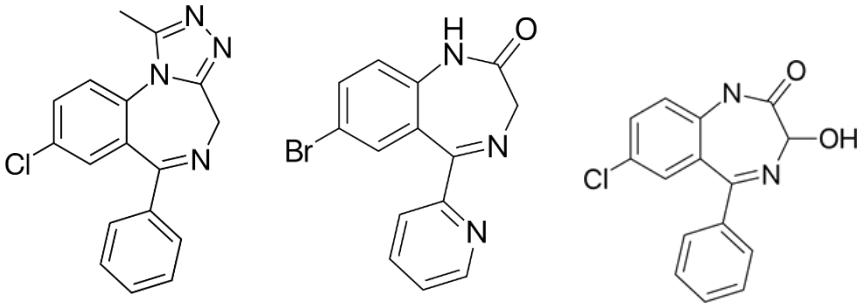
Pentobarbital



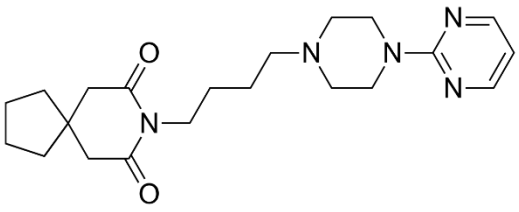
Thalidomide



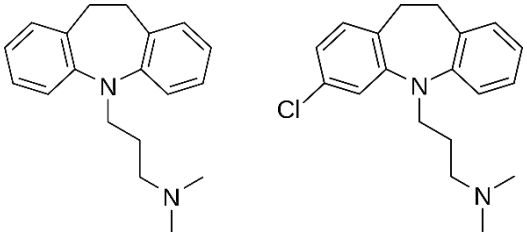
Meprobamate



Three of the most commercialised anxiolytic benzodiazepines
(Left: alprazolam; middle: bormazepam; right: oxazepam)



Buspirone



Tricyclic antidepressants
(Left: imipramine; right: clomipramine)

FIGURE 3.11 – Overview of some past and current anxiolytic molecules.

TABLE 3.2 – Demographic repartition of anxiolytic BZD-treated subjects in France in 2011 and 2012.

Adapted from (ANSM, 2013).

	2011 (*n* = 56,625)	**2012 (*n* = 57,060)**
Mean age, years (SD)	55.2 (18.3)	55.5 (18.3)
Median age, years (IQR)	55 (42–55)	55 (42–69)
Age, % (*n*)		
< 18 years	1.5 (855)	1.5 (847)
18 to 35 years	13.6 (7,715)	13.5 (7,689)
35 to 50 years	25.9 (14,687)	25.7 (14,648)
50 to 65 years	29.1 (16,492)	29.1 (16,605)
65 to 80 years	19.9 (11,265)	19.7 (11,255)
> 80 years	9.9 (5,611)	10.5 (6,016)
Women, % (*n*)	67.2 (38,062)	66.6 (38,020)

Anxiety disorders are then described as a human phenomenon. However, to better understand the underlying behavioural and neural mechanisms, animal models have been developed over the past decades to mimic these disorders and understand the functioning of the CNS.

4 RODENTS' MODELS OF ANXIETY

Contents

This section will then first aim to present some of the classically used animal models to reproduce human anxiety disorders (cf. ***4.1***). The second part will focus on a specific tool to study neuronal activity in rodents: the c-Fos protein (cf. ***4.2***).

4.1 Anxiety and animals

Even though we have described anxiety mostly in humans (cf. ***3.1*** and ***3.3***), the question remains to understand whether this phenomenon has an equivalent amongst other animals, and specifically amongst mammals and more particularly rodents. Some were sceptical or particularly timid on their answers to that question.

Consequently, Cassano described in 1983:

> fear is a primitive state of mind found throughout the animal kingdom, whereas anxiety is part of conscious experience and takes shape as a typically human function or attitude. Thus, the age of anxiety could be said to begin with the emergence of Homo sapiens. In anxiety, unlike fear, there may be no threatening situation at all, or only a vague one.

One of the many arguments on that matter of the non-believers is that anxiety is based on the notion of future, as seen the definitions proposed at the beginning of this section (cf. ***3.1.1*** and ***3.1.2***), which is supposed to be a human concept. Likewise, Lord Russell Brain, yet being a fervent defender of the existence of a pain concept in the animal reign, considered in 1963 that the nervous system of these latter was too little developed for experiencing complex emotions such as anxiety.

Official texts also came down on that question. In this way, the U.S. Department of Agriculture (USDA) stated in 1971 ‘the word anxiety is a psychiatric term that is only applicable to humans’. ‘Anxiety’ has since then been replaced by ‘distress’, ‘which is more descriptive of the physical visible state of animal’ (Animal and Plant Health Service 1971, p. 919).

However, behavioural and physiological responses precisely described in humans may also be observed in animals. In 1915, Walter Cannon coined the term ‘fight or flight’ for animals in his *Bodily Changes in Pain, Hunger, Fear and Rage: An Account of Recent Researches into the*

Function of Emotional Excitement. If the threat (predator most of the time) is close to the animal, the latter will initiate a defensive attack ('fight'), but if the threat is far enough the animal will be most likely to flee ('flight'; a freezing response can be observed if escape cannot be performed) (***FIGURE 4.1***). These behaviours are accompanied by several physiological changes: motor tension – shakiness, jumpiness; autonomic hyperactivity – sweating, pounding heart, increased pulse rate and respiration, frequent urination, diarrhoea; apprehensive expectation – inhibition of behavioural repertoire in novel situations; hyper attentiveness – vigilance, scanning (DeGrazia and Rowan, 1991), all these behaviours having already been described in humans' anxiety disorders (cf. ***3.1.1*** and ***3.3***). Eventually, the principal argument of the existence of an anxiety phenomenon within animals lies in the fact that they are sensitive to both anxiolytic and anxiogenic agents (Ninan *et al*, 1982). This will be described later in this section.

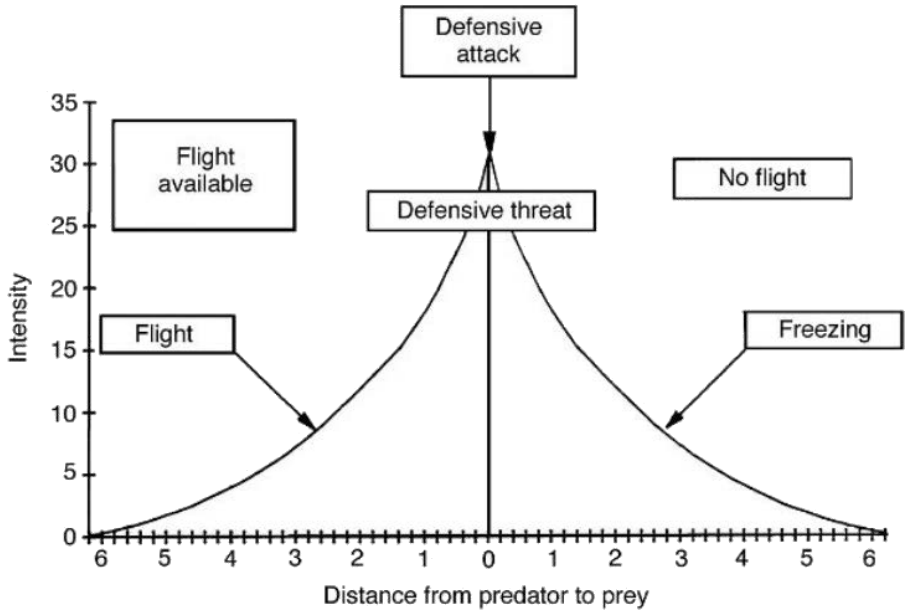


FIGURE 4.1 – The fight and flight system according to the defensive distance.

Another classification of defensive behaviour in a dangerous situation according to the distance (arbitrary unit) separating the individual (here a prey) from the threat (here a predator) (Gray, 1982).

To sum up, it thus exists similarities in behavioural and physiological responses between patients diagnosed with anxiety disorders and individuals, both humans and animals, placed in anxiety inducing situations. By applying Morgan's canon – 'In no case is an animal activity to

be interpreted in terms of higher psychological processes, if it can be fairly interpreted in terms of processes which stand lower in the scale of psychological evolution and development' – it then can be concluded that 'human anxiety, or something very like it, exists also in animals…' (Gray, 1982). The complexity to study this phenomenon in animals lies in the fact that, unlike humans, they do not speak to communicate.

Two trails have then been investigated: behavioural models that appeared in the second half of the 20th century, and complementary genetic models, which appeared at the really end of the 20th century. According to William McKinney, an animal model is an experimental situation in a given species in order to study a phenomenon occurring in another species (1984). Kaplan was already specifying in 1973 that an animal model is valid if and only if it has the same structure of the human behaviour or pathology. Different criteria have then been introduced to set boundaries to animal models (cf. ***TABLE 4.1*** and ***FIGURE 4.2***) (Belzung and Lemoine, 2011).

TABLE 4.1 – The criteria of validity for animal models.

Adapted from (Belzung and Lemoine, 2011).

Kind of validity	Aspect of validity	Object of validity
Homological validity	**Species validity**	Species
	Strain validity	Strain
Pathogenic validity	**Ontopathogenic validity**	Interaction transforming an initial organism into a vulnerable organism
	Triggering validity	Interaction transforming an initial or a vulnerable organism into a pathological organism
Mechanistic validity		Theoretical cognitive or neurobiological mechanisms producing the observable effects of the disease
Face validity	**Ethological validity**	Behavioural symptoms of the disease
	Biomarker validity	Biomarkers associated with the disease
		relation
Predictive validity	**Induction validity**	Relation between the triggering factor and the observable effects of the disease
	Remission validity	Relation between the therapeutic agent and the observable effects of the disease

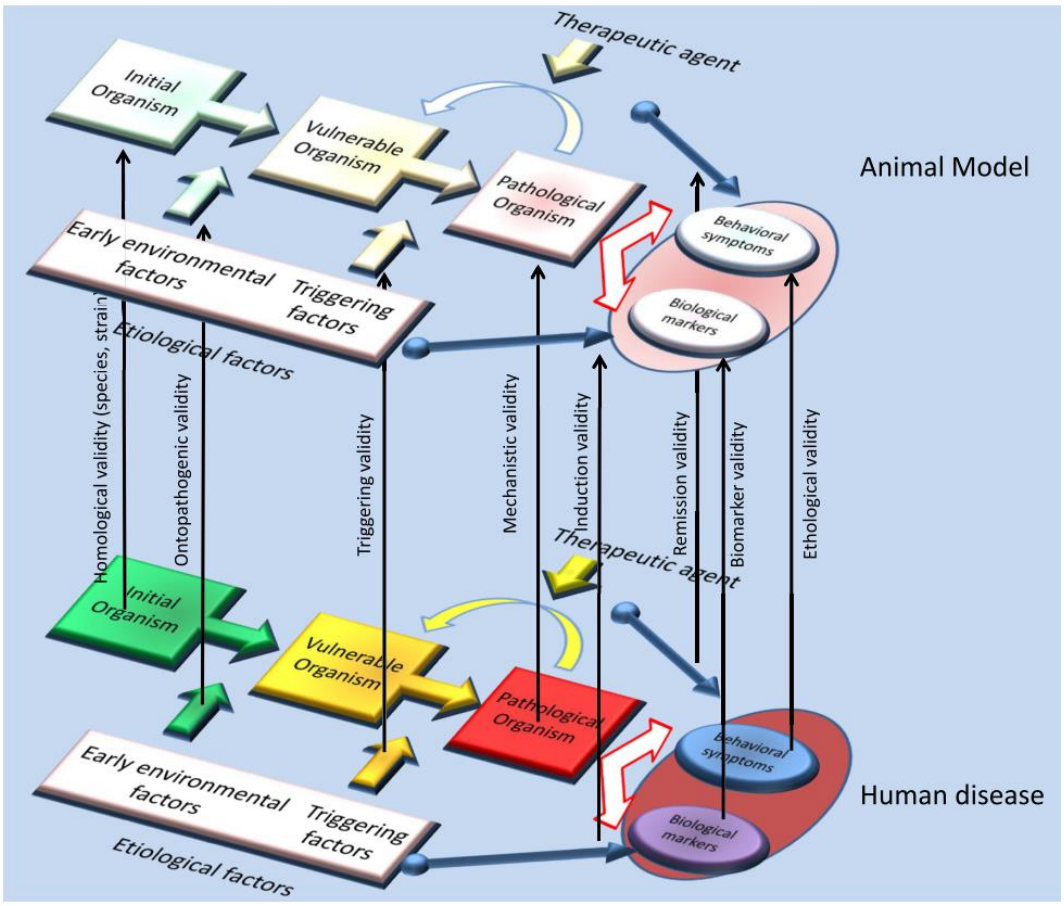


FIGURE 4.2 – The criteria of validity for animal models.

The different criteria linking a human disease (at the bottom) with an animal model (at the top). Every step from the initial healthy organism (on the left) to the pathological organism and its outcomes (on the right) can be taken into account (Belzung and Lemoine, 2011).

4.1.1 Behavioural models

As it is not possible to exactly reproduce the specific human anxiety disorders due to their aetiology (cf. ***3.3.1***), behavioural models developed in the second half of the 20th century reproduced situations triggering high anxious states in controlled experimental situations (Steimer, 2011). These models are the closest to the definition of state-anxiety (the anxiety felt in a given situation, cf. ***3.1.3***).

As seen overhead, behavioural models have to respect a few criteria to assess the validity of these models in regard to human anxiolytic disorders. Four criteria are commonly defined and retained for these models (Belzung and Griebel, 2001; Bourin, 2015; Millan, 2003):

- **Face validity** (isomorphism model): the behavioural and physiological responses of the animal must be related to that of humans. Despite some differences in the modality of the response to the threatening stimulus, the goal has to be the same (e.g. running, flying, or swimming away from the danger). This criterion might be tricky to evaluate, due to the absence of speech amongst animals.
- **Ecological validity**: this criterion is a consequence of the previous one. The pertinence of the threatening stimulus and the possibility to express an adapted behavioural response have to be coherent with the natural environment of the studied animals.
- **Construct validity** (homologous model): neurological and psychological mechanisms underlying the anxiety related behaviours have to be the same between humans and animals. Indeed behaviours are events and processes at the same time: the observed behaviour is the result of the integration of all the processes, which took places in organs in interaction with external sociological and physical stimuli. This criterion is important for the understanding of the neurobiology of anxiety (cf. ***3.2***).
- **Prediction validity** (correlational model): clinically efficient anxiolytic and anxiogenic drugs can be detected using the model. Consequently, molecules that do not have any impact on human anxiety should not alter the model. This criterion has suffered some difficulties recently as most of the models were tested using benzodiazepines and since then new clinically efficient anxiolytics (such as buspirone and antidepressants) were not detected suing the same models.

Behavioural tests are traditionally distinguished between unconditioned and conditioned tests (***TABLE 4.2***) (Belzung and Griebel, 2001; Bourin, 2015; Campos *et al*, 2013; Millan, 2003; Steimer, 2011). **Unconditioned tests** are based on the exploration of novel places with specific characteristics (e.g. elevated, brightly lit or enclosed) while **conditioned tests** are based on a noxious stimulus, which trigger the anxiety behaviours. Some parameters studied in tests are strongly reliant on the locomotion of animals but not their anxiety levels (Belzung and Le Pape, 1994) and some tests used in anxiety evaluation are also used to evaluate the locomotion of

animals to detect sedative properties of molecules for instance (e.g. open-field test) (Sestakova *et al*, 2013).

As an example, the **light/dark box** is one of the first ethological tests to be introduced to study anxiety in rodents (Crawley and Goodwin, 1980). It consists of two separated compartments, one is brightly lit while the other is darkened, that animals can freely explore. Face validity is respected as light serves here as an anxiogenic stimulus and there is a conflict between the aspiration to explore and the aspiration to dodge the brightly lit compartment (Lister, 1990). Different parameters can be scored during 5-min sessions: latency to enter the unknown compartment, transitions between the two compartments, time spent in the lit compartment, rears in the lit compartment (Bourin and Hascoët, 2003; Hascoët *et al*, 2001; Hascoët and Bourin, 1998). The more time the animals spent in the lit compartment, the less anxious they are thought to be. Moreover, drugs that increase the number of transitions without impacting the locomotor activity of animals are said to be anxiolytic, confirming thus the prediction validity (Crawley, 1981; Crawley and Goodwin, 1980).

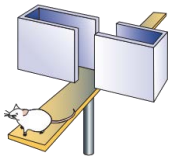
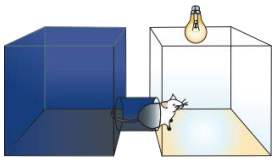
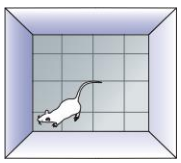
Another example of unconditioned test is the **open-field** (Wilson *et al*, 1976). This test consists again in placing an animal in an unknown device where it cannot escape. Rodents tend to spend more time in the periphery of the device, ambulating close to walls, than in the centre of it (thygmotaxic behaviour). The ratio 'number of squares visited in centre/number of squares visited on periphery' is then a good marker to evaluate the anxiety of animals: the lower it is, the more anxious the animals are (Bourin *et al*, 2007). This test has however a limit: it is often used as a locomotor test by several laboratories to investigate the sedative effects of some compounds (Dela Peña *et al*, 2016; Sestakova *et al*, 2013). It is then sometimes hard to differentiate the locomotor and the anxiety component of this test. Another limit is the specific absence of a standardised device: different devices have indeed been presented in the literature, either circular or rectangular, lit or not, … (Bourin *et al*, 2007)

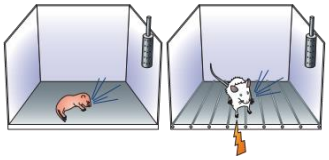
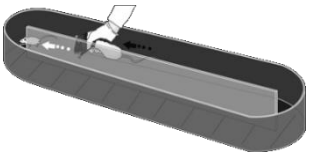
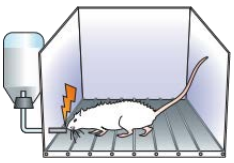
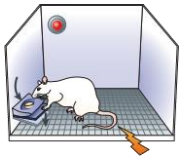
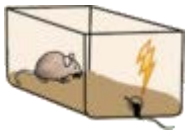
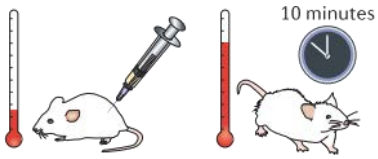
Eventually, an example of a conditioned model could be the **conditioned defensive burying test (CDB)**. It is based on a stereotyped rodents' behaviour: they bury potentially harmful objects by projecting material (e.g. pine needles, sand, sawdust, faeces) with their forepaws. The CDB, strictly speaking, was introduced in 1978 (Pinel and Treit, 1978): it consists of a chamber in which animals are habituated by repeated exposure; the day of the test, a probe is introduced in the chamber. Once the animal touches the probe with its forepaws, it receives a

brief shock and starts then to bury the probe, which is now associated as an aversive stimulus. The duration of burying is related to the anxiety level of the animal (Treit *et al*, 1981). The predictive criteria is respected as anxiolytic drugs, such as benzodiazepines (cf. **2.2**), buspirone, or tricyclic antidepressants, decrease the duration of probe burying (De Boer and Koolhaas, 2003; Treit *et al*, 1981).

TABLE 4.2 – Most commonly used behavioural tests to study anxiety in rodents.

GAD, generalised anxiety disorder; PTSD, post-traumatic stress disease; SAD, social anxiety disorder. Adapted from (Belzung and Griebel, 2001; Bourin, 2015; Campos *et al*, 2013; Griebel and Holmes, 2013; Millan, 2003; Steimer, 2011).

Test	Anxiety disorder	Year of introduction	Number of utilisations between 1960 and 2012	Reference
UNCONDITIONED TESTS				
Exploration behaviours (approach avoidance behaviours)				
Elevated plus-maze 	GAD	1984	2,565	(Handley and Mithani, 1984)
Light/dark box 	GAD	1980	846	(Crawley and Goodwin, 1980)
Open-field 	GAD	?	547	(Denenberg, 1969)
Social behaviours				
Social interaction 	GAD, SAD	1980	808	(File, 1980)

Ultrasonic distress vocalisations 	GAD	1985	510	(Gardner, 1985)
Antipredator behaviours				
Mouse defence test battery 	GAD, panic disorder, PTSD	1997	N/A	(Blanchard *et al*, 1997)
CONDITIONED TESTS				
Conflict procedures				
Vogel 	GAD	1971	592	(Vogel *et al*, 1971)
Geller-Seifter 	GAD	1962	295	(Geller and Seifter, 1962)
Non-conflict procedures				
Conditioned defensive burying 	GAD	1981	N/A	(Treit *et al*, 1981)
Stress-induced hyperthermia 	GAD	1990	N/A	(Lecci *et al*, 1990)

Despite the great usefulness of the overhead-mentioned tests, they suffer from different limits. Indeed, the behavioural tests evaluate mostly the generalised anxiety disorder as defined by the DSM-V: excessively anxiety in several situations (***TABLE 4.2*** and cf. ***3.3.1***). The anxiolytic drugs screened on these test may then not be efficient on other specific anxiety disorders (e.g. panic disorders or specific phobia). Other tests have then been proposed to fill these gaps. Exposition to a predator odour may be a good animal phobia model (Dielenberg *et al*, 2001a, 2001b; Dielenberg and McGregor, 2001). The defence test battery introduced by Blanchard, which puts rodents in the presence of a predator in an unescapable area, could be a good model for panic disorders depending on the behaviours scored (Blanchard *et al*, 1990, 1997, 2001; Blanchard and Blanchard, 2003; Griebel *et al*, 1995; Moreira *et al*, 2013). Eventually, the marble burying test, the action of rodents to bury unknown objects (such as marbles) in sawdust, is thought to be a good model for obsessive-compulsive disorders (Egashira *et al*, 2013; Njung'E and Handley, 1991). A good way to screen anxiolytic compounds is finally to test them within different behavioural tests (Ramos, 2008). Some authors also proposed to combine the open-field, the elevated plus-maze and the light/dark box into one unique test (Ramos *et al*, 2008).

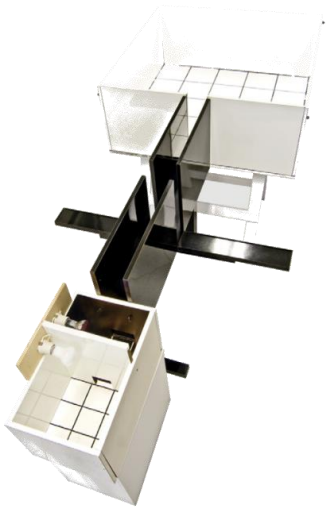


FIGURE 4.3 – The integration of three widely used tests of anxiety.
This test combines the open-field (at the top), the elevated plus-maze (in the middle) and the light/dark box (at the bottom). Animals are placed at the centre of the open-field and behaviours are scored for 15 minutes (Ramos *et al*, 2008).

Another great pitfall of these tests is the one-trial tolerance effect (File, 1990). Re-exposure to a behavioural test deeply affects the behaviours of an animal due to the habituation to the task (Rodgers and Shepherd, 1993). It is then greatly recommended that animals be only tested once in a given test. New procedures are then being tested to overcome this effect, such as using the triple test presented overhead (Hussin *et al*, 2012)

4.1.2 Trait models

Different methods are used to evaluate trait-anxiety amongst rodents (Belzung and Griebel, 2001; Millan, 2003): deletion of specific genes involved in anxiety regulation, such as the serotonin type 1A receptor (Finn *et al*, 2003; Griebel and Holmes, 2013), or selection of animals based on their basal anxiety levels, for instance. The following paragraph will only focus on the latter method. However, despite using these different methods, it has to be noted that anxiety levels still need to be evaluated using the behavioural models described above (cf. ***4.1.1***).

The use of specific inbred or outbred strains, which elicits different levels of basal anxiety can be an example, as mice strains do not perform the same behavioural responses (van Bogaert *et al*, 2006; Crawley *et al*, 1997). As far as mice are concerned, C57Bl6 and Swiss strains are recommended for anxiety evaluation, due to their high exploratory behaviour (Bourin and Hascoët, 2003; Crawley and Davis, 1982; Misslin *et al*, 1989). Amongst these specific strains, the selection of specific individuals with high or low basal levels of anxiety can also be performed (Landgraf and Wigger, 2002; Liebsch *et al*, 1998; Salomé *et al*, 2004). Eventually, it has been shown that females were more sensitive to anxiety-inducing situation than males (Dalla *et al*, 2005; Palanza, 2001), but the influence of menstrual cycles on both fear and anxiety associated behaviours make them harder to study (Basso *et al*, 2011; Díaz-Véliz *et al*, 1997; Galeeva and Tuohimaa, 2001; Gouveia *et al*, 2004).

Labs conditions also have an impact on the basal levels of anxiety in rodents. In this way, repeated handling, and especially tail picking, increased anxiety levels in mice compared to undisturbed animals (Balcombe *et al*, 2004; Gadek-Michalska and Bugajski, 2003; Hurst and West, 2010; Longordo *et al*, 2011), indicating that mice do not habituate to daily handling compared to rats (Biggio *et al*, 1990). The type of housing also impact on mice behaviours, separate housing is believed to be more anxiety-inducing that group housing (Liu *et al*, 2013). However, these results may be discussed depending on the conditions of the study (Hunt and

Hambly, 2006). More anecdotally, male experimenters have been proven to increase the anxiety levels of rodents in lab conditions (Bateson, 2014; Sorge *et al*, 2014).

The advances in animal modelling of anxiety disorders allowed a better understanding of both the neurological mechanisms underlying the anxiety phenomenon (cf. ***3.2***) and the pharmacology and therapeutic targets to deal with the associated disorders (***FIGURE 4.4***).

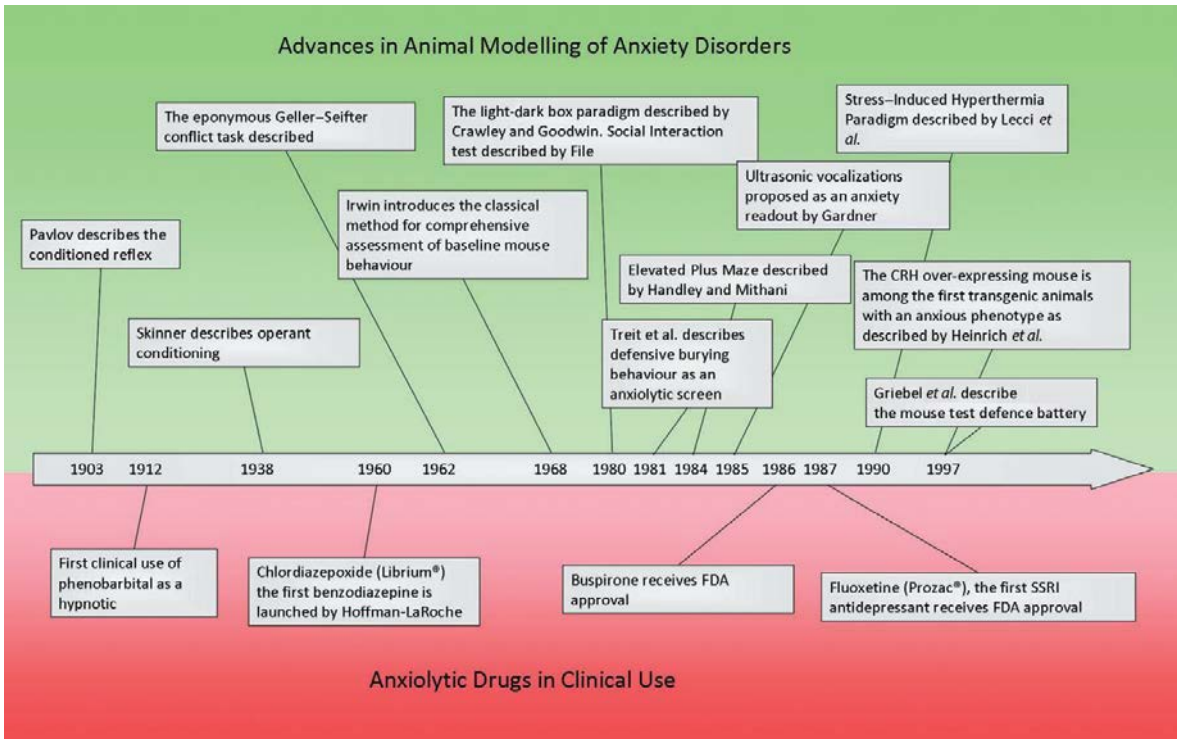


FIGURE 4.4 – Advances made in the modelling of anxiety disorders in humans compared to the introduction of novel anxiolytic drug classes across the past century.
(Cryan and Sweeney, 2011).

4.2 An example of brain activity evaluation tool: the c-Fos protein

Several techniques have been developed to study brain activity in both humans and rodents. Electrophysiology and, in a larger way, electroencephalography (EEG) are direct measures of neuronal activity *via* the recording of electric currents or electric fields. Magnetoencephalography (MEG) is similar to EEG but detects the magnetic component of electrical neuronal activity. Even if all these methods register direct neuronal activity, they all

suffer from a bad spatial resolution, electrophysiology registering the activity of a single neuron while EEG/MEG are mostly limited to cortical activity. Some more precise indirect methods have then been set up. While PET scans and fMRI are amongst the gold standard of brain activity evaluation in clinical studies, the direct labelling of activated neurons with specific neuronal activity marker in rodents allows the best spatial resolution of all the methods named above. We will then now focus on the labelling of neuronal activity *via* immunohistochemical techniques, and specifically *via* the detection of the c-Fos protein.

4.2.1 c-Fos, marker of neuronal activity

The **c-Fos protein** is a 380 residue (55 kDa) transcription factor (Kovács, 1998). It also belongs to the "immediate early-genes" family, which is triggered by extracellular stimuli (other examples are Egr1/Zif268, Arc/Arg3.1…). Thus, c-Fos possesses a few characteristics that allow it to be used as a marker of neuronal activity:

- **c-Fos expression is low in the intact brain under basal condition** (Herrera and Robertson, 1996; Kovács, 1998, 2008).
- **c-Fos is stereotypically induced in response to several extracellular signals** (Kovács, 2008). Intracellular calcium influx after a depolarisation mediates synaptic activity to *c-fos* gene expression, *via* the MAPK (mitogen-activated protein kinase) pathway. As MAPK is slow and requires strong external stimulation and higher calcium influx, weak stimuli are less likely to be translated into c-Fos expression (Chung, 2015). To maximise the signal, a novel stimulus is often recommended, while, in contrast, repetitive stimulations are expected to diminish the signal.
- **c-Fos response is transient.** Maximal level of the c-Fos protein is attained between 1 and 3 hours after the stimulation and c-Fos gradually disappears 4 to 6 hours after the stimulus (Kovács, 1998). This may be due to the instability of *c-fos* mRNA and the auto-repression of *c-fos* transcription (Chung, 2015).

However c-Fos use as a marker of neuronal activity raises a few cons. Indeed, c-Fos detection gives no idea of the nature of activated neurons and a labelling with another marker is sometimes needed to precise the nature of them. Some regions differentially express c-Fos, some needing a strong stimulus (e.g. visual cortex), other needing a mild stimulus (e.g. amygdala) and finally some regions do not express c-Fos at all (e.g. substantia nigra)

(Dragunow and Faull, 1989; Kovács, 1998). Eventually, there is an hypothesis that c-Fos could also be considered as a learning marker, due to the fact that repetitive positive stimuli (such as cocaine consumption) increase c-Fos expression over repetition, while repetitive negative stimuli (such as social defeat) decrease c-Fos expression over repetition (Chung, 2015).

4.2.2 c-Fos in the study of anxiety

c-Fos expression has then been extensively used to study anxiety. A few studies investigated the effect of different physical and emotional challenges on c-Fos expression in several brain regions (***TABLE 4.3***). As far as anxiety is concerned, two studies described the effect of the elevated plus-maze (cf. ***4.1.1***) demonstrating that this test raise c-Fos expression in different brain regions involved in anxiety regulation, including prefrontal cortex, hippocampal formation and amygdala (Duncan *et al*, 1996; Silveira *et al*, 1993).

This technique has also been used to study the effects of different anxiolytics on neural activity. Some examples can then be cited with diazepam. A study by de Medeiros and colleagues using rats in a restraint stress protocol demonstrated that a diazepam treatment decreased c-Fos expression in hippocampal formation, and BNST while increasing its expression specifically in the central nucleus of the amygdala (de Medeiros *et al*, 2005). Another study from Lkhagvasuren and colleagues showed that a diazepam treatment reversed the increased c-Fos expression induced by a social stress protocol in the entire brain, while increasing it again in the central nucleus of the amygdala. Results may differ between studies as there is dose-response effect of diazepam (Beck and Fibiger, 1995) and different challenges used to trigger c-Fos expression induced different c-Fos patterns (Kovács, 1998).

Other anxiolytic-like molecules have been tested using the c-Fos protocol. As an example, yokukansan, a traditional herb medicine, decreased the augmented c-Fos expression observed after a restraint paradigm in the central and basolateral nuclei of the amygdala, while having no decreasing effects in different hypothalamic nuclei (Shoji and Mizoguchi, 2013). Another example could be lavender oil, an essential oil that have anxiolytic-like properties (de Sousa *et al*, 2015), which has no effect on c-Fos expression in the accumbens nucleus, nor the amygdala, but decreases its expression induced by an open-field test in hypothalamic nuclei (Shaw *et al*, 2011).

TABLE 4.3 – Summary of c-Fos induction following emotional and physical challenges.

BNST, bed nucleus of the stria terminalis; PAG, periaqueductal grey; PVN, paraventricular nucleus of the hypothalamus; NTS, nucleus of the tractus solitarius. Adapted from (Kovács, 1998).

Region	Air puff	Restraint	Immobil	Foot shock	Swim	Plus-maze	Aggression	Fear	Audio	Ether	Pain
					Cortices						
Frontal cortex	+/–	+	+	++	++	++	+/–	+	0	++	+
Cingulate cortex	+/–	++	++	++	+++	++	+	++	+++	++	+
Orbital cortex	0	+++	+	+	++	++	0	+	++	+/–	
Pyriform cortex	0	+++	++	+++	+++	++		+/–	++	+++	+
Hippocampal formation	0	+	++	+	+	+/–		+	+/–	0	
					Hypothalamus						
PVN parvocell	+/–	++	+++	++	++	+/–	+	++	++	+++	++
PVN magnocell	0	+	+/–	+/–	+	0				0	
Supraoptic	0	0	+	+	+/–	0	0			0	
Dorsomedial	0	+	+	+		+	+	+/–	+	+	
Ventromedial	0	+	+	0		+/–	+	+	0	+/–	
					Amygdala						
Basolateral	0	+	+	++	+	+			+/–	+	++
Central	0	+/–	+	+/–	+/–	+/–	+	++	0	+	
Cortical	0	+	+	++	+++	+	0	0	+	0	
Medial	+/–	++	+	++	+++	+	++	++	+	+	+
BNST	+/–	+	++	+/–	+	+	+	+++	++	++	+
Lateral septum	+/–	++	++	+	++	++	++	+/–	++	+++	+
PAG	+	+	+	++	+	+	++	+	+	+	++
Locus coeruleus	0	+	+	++	++	+	+	++	+	+	+++
NTS	0	++	++	++	++	+/–	+			++	++

PART II – PERSONAL WORK

1 OBJECTIVES OF THE RESEARCH PROJECT

The objective of this thesis is to understand better the cerebral mode of action of α-casozepine (α-CZP). More specifically, this thesis will focus on the effect(s) of α-CZP on neural activity: the aim was to characterise the changes in the activity of the brain areas involved in the modulation of anxiety after a unique intraperitoneal administration of α-CZP. As α-CZP has been characterised as a benzodiazepine-like peptide (cf. ***2.3.1*** and ***2.4.1***), diazepam, a reference benzodiazepine, was used in parallel to compare the results obtained between the two molecules.

Different studies have reported a better effect of the hydrolysate (CH) amongst anxious individuals. Indeed, a study conducted on rats, pointed out the fact that the sleep modulating effects of CH were only observable when the rats were submitted to a chronic mild stress (Guesdon *et al*, 2006). Palestrini and colleagues revealed that an iCH-supplemented diet positively affected behavioural and physiological parameters amongst anxious dogs, while the same diet seemed to have less effect in non-anxious dogs (Palestrini *et al*, 2010). Concerning clinical studies, responses to the anxiolytic-like effects of CH were different between low- and high-stress individuals. A first study conducted amongst individuals submitted to a Stroop test (***FIGURE 2.9***) exposed that a supplementation of 150 mg/day was efficient to decrease the augmentation of blood pressure triggered by the test after 30 days in the global population. However, this effect could be observed after only 10 days of supplementation amongst high-stress individuals (Lanoir *et al*, 2002). Eventually, a second clinical study displayed also a stronger effect of CH after a 30 days supplementation amongst high-stressed women using a self-reported questionnaire (Kim *et al*, 2007).

In order to better understand the anxiolytic-like effects of α-CZP in a murine model, the separation of low- and high-stress individuals may then be interesting to **determine if there is a specific response amongst high-stress individuals**. Two possibilities will then be conducted:

- the separation of animals according to their basal anxiety levels (trait-anxiety models, ***4.1.2***) using an open-field device.
- the separation of animals into two different situations: a control situation, where animals are left undisturbed in their housing conditions, and an anxiety-inducing

situation (state-anxiety models, ***4.1.1***), where animals are placed in a behavioural test, such as the light/dark box.

A global approach of α-CZP action on neural activity was carried out. As seen above, anxiety is a complex phenomenon that is regulated by several brain areas acting together (cf. ***3.2***). Studying a single area would then not be relevant. The study of an entire mouse brain hemisphere, using sagittal sections, will then give us the opportunity to investigate different brain regions involved in anxiety regulation at the same time. Comparison can then be made between the administration of α-CZP, a vehicle that can act as a negative control, and diazepam, a reference benzodiazepine, acting as a positive control. Depending on the results, some may even be confirmed using coronal sections under the same experimental conditions. Neural activity was evaluated by labelling neuronal activity, using c-Fos immunofluorescence in mice brains (cf. ***4.2***).

As derivatives of α-CZP may also participate to its anxiolytic-like properties (cf. ***2.4.2***). It can be hypothesised that α-CZP and its derivatives may not act the same on the neural activity. **A comparison between the action of α-CZP and one of its derivatives found by *in vitro* digestion (YLGYL) on neural activity**, using the same experimental paradigm as before, can then bring light on that hypothesis.

2 EVALUATION OF THE MICES BASED ON THEIR BASAL LEVELS OF ANXIETY

2.1 Introduction

Anxiety is the reaction of an individual to an upcoming threat. In the 1960s, Spielberger developed a multifactorial theory of anxiety, making the distinction between the reaction to a threat (state-anxiety) and the predisposition to respond to threat (trait-anxiety) (Spielberger, 1966). It has since been stated that individuals with higher trait-anxiety levels displayed higher state-anxiety levels in a given situation (Graziani, 2008). Evaluating the trait-anxiety level of an individual could then allow us to select the ones with high basal anxiety levels.

Several models have been develop to mimic trait-anxiety in rodents exploring different methods: behavioural models, inbreeding models, genetic models (Belzung and Griebel, 2001), … Behavioural models are easier to set up. By evaluating the basal anxiety levels of animals, i.e. without any treatment of specific conditions, animals can be discriminated as either high (HAB) or low (LAB) anxiety-related behaviours individuals (Salomé *et al*, 2004). This discrimination is commonly used in rats models but a lot less amongst mice (Landgraf and Wigger, 2002).

One of the most frequently used ethological tests is the open-field (Griebel and Holmes, 2013), which consists of an unescapable arena where the rodent is put for 5-min sessions. The more anxious the animal is, the more time it will spend in the periphery zone, and consequently the less time in the centre zone (Liebsch *et al*, 1998). This study will then focus on whether it is possible or not to discriminate Swiss mice on their basal anxiety levels using an open-field test.

2.2 Material and methods

2.2.1 Animals

All experiences were approved by the French Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt on the recommendation of the Comité d'Ethique en Expérimentation Animale of Jouy-en-Josas (N°02237.01). Male Swiss mice (Janvier Labs, France), aged of 9 weeks old at the reception, were received in five different batches between

February 2014 and February 2016. Number of mice per batch were the following: Batch #1: 32 mice, Batch #2: 48 mice, Batch #3: 36 mice, Batch #4: 32 mice, and Batch #5: 48 mice (n_{total} = 196 mice). Mice were housed individually and maintained on a 12 h inversed light/dark cycle (lights off at 08:00 a.m.) and controlled environment (temperature 22±1°C, humidity 60%). Water and food were available ad libitum. Mice were handled every day by the same experimenter and particular care was taken to limit any kinds of external stress (variations in light, noise or odour). Behavioural tests were performed during their active period (08:00 a.m. – 08:00 p.m.).

All batches were put in the exact same conditions in order to minimise the impact of lab conditions on animals' behaviours. After a week of acclimation, mice received a soy protein-based diet. One week later, their basal anxiety levels were evaluated with an open-field device.

2.2.2 Open-field

This device consists in a 40 × 40 cm arena with 20 cm high walls where the animals cannot escape (***FIGURE 2.1***). Behavioural tests were conducted under a dim red light that rodent cannot perceive but allowed the recording of behaviours. Mice were individually tested in 5-min sessions. They were put in the centre of the apparatus to start the test session. Behaviour was recorded using a camera (Logitech, Switzerland), while the experimenter was waiting outside of the room in order to not disturb the behaviour of the mice. The transitions in the centre and the transitions in the periphery were recorded under blinded conditions using The Observer XT 8.0 (Noldus, The Netherlands). Three parameters were scored: the number of entries in the centre of the device, the percentage of time spent in the centre and the total number of transitions, as this test is dependent on the locomotor activity of the mice.

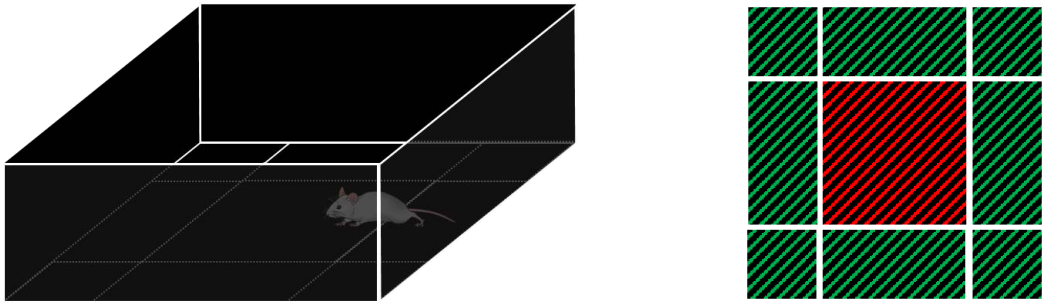


FIGURE 2.1 – Diagram of the open-field.

Left: side view.

Right: Top view, centre in red, periphery in green.

2.2.3 Statistical analysis

Data were evaluated with one-way analysis of variance (ANOVA). Multiple comparison analysis was performed with Bonferroni post-hoc tests using R (R Core Team, 2013). All data are reported as mean ± SD. Differences were considered to be significant at the $p < 0.05$ level.

2.3 Results

A total of 182 mice were finally analysed (about 93%) as some animals managed to jump out of the device. Final number of animals per batch were as follows: Batch #1: 29 mice, Batch #2: 39 mice, Batch #3: 36 mice, Batch #4: 31 mice, and Batch #5: 47 mice.

Some differences were found between the different batches according to the parameters scored (***FIGURE 2.2***). No difference was found for the total number of transitions between batches. However, animals from Batch #1 and Batch #2 made more entries in the anxiety-inducing centre compared to animals from Batch #3 and Batch #4, while animals from the Batch #2 spent more time in the centre compared to the other batches.

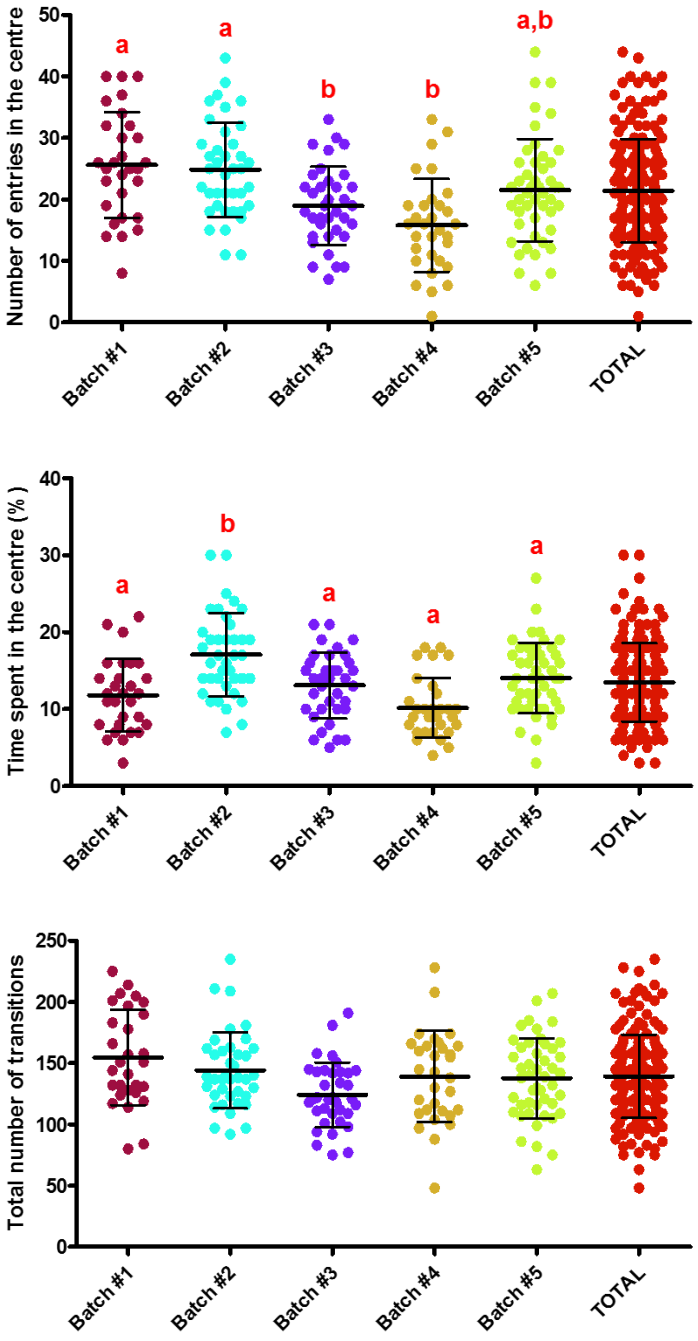


FIGURE 2.2 – Parameters scored in the open-field for each batch of mice (#1 to #5).

Top: number of entries in the centre

Middle: time spent in the centre/time spent in the periphery

Bottom: total number of transitions

Means with different letters are significantly different (Bonferroni post-hoc, $p < 0.05$)

All batches were then pooled to evaluate the repartition of animals giving to the different parameters scored in the open-field (***FIGURE 2.3*** and ***SUPPLEMENTARY DATA 2.1***). The number of entries in the centre and the total number of transitions followed a normal distribution according to the Shapiro-Wilk test (p = 0.08926 and p = 0.2292, respectively). On the other hand, the time spent in the centre did not follow a normal distribution (p = 0.009572), and more precisely due to the animals coming from Batch #4 (p = 0.009885).

2.4 Discussion

According to these results, animals have the same basal level scores of anxiety between different batches. Only animals coming from Batch #2 could be counted as less anxious than the animals coming from other batches. Despite this small heterogeneity between batches, mostly all parameters followed a normal distribution with all the batches taken together. In this way, no bimodal distribution was observed for the studied parameters, compared to what can be observed in previous papers discriminating animals on their trait-anxiety levels (Liebsch *et al*, 1998; Salomé *et al*, 2004; Yen *et al*, 2013).

A first difference to be noted was that animals on these studies were specifically bred over generations to only keep animals with extreme reactions in anxiety evaluation tests. Ours were directly coming from the supplier and were not specifically selected on their behavioural results. Moreover, even if this type of selection has been used in mice, it is more widely used in rats in the literature. The choice of the strain also have an impact on the basal anxiety levels of animals as Swiss mice have been characterised as being less anxious as other strains such as the C57Bl/6 (van Bogaert *et al*, 2006). However, the use of outbred strains, such as Swiss mice, can be a better model of a genetically diverse population to test new health-impacting products.

Other parameters have been proposed in the literature to evaluate anxiety in the open-field test, e.g. the ratio ‘number of transitions in the centre/number of transitions in the periphery’ or ‘time spent in the centre/time spent in the periphery’ (Bourin *et al*, 2007). However, they are highly correlated with two of the parameters scored in this study (***SUPPLEMENTARY DATA 2.2***).

As these results did not allow us to select animals on their basal anxiety levels, another way to artificially create high- versus low-anxiety animals is to put part of them in an anxiety-inducing situation to increase their state-anxiety.

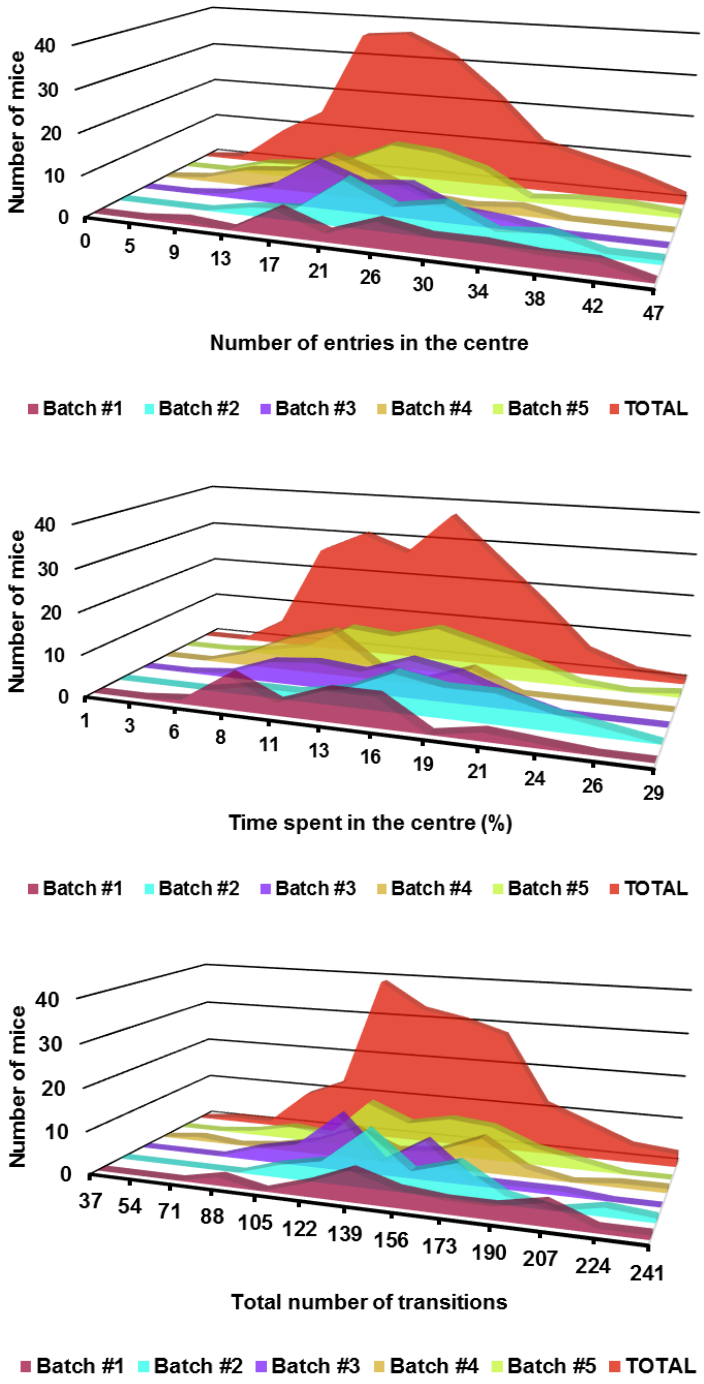


FIGURE 2.3 – Repartition of mice according the parameters scored in the open-field.

Top: number of entries in the centre

Middle: time spent in the centre/time spent in the periphery

Bottom: total number of transitions

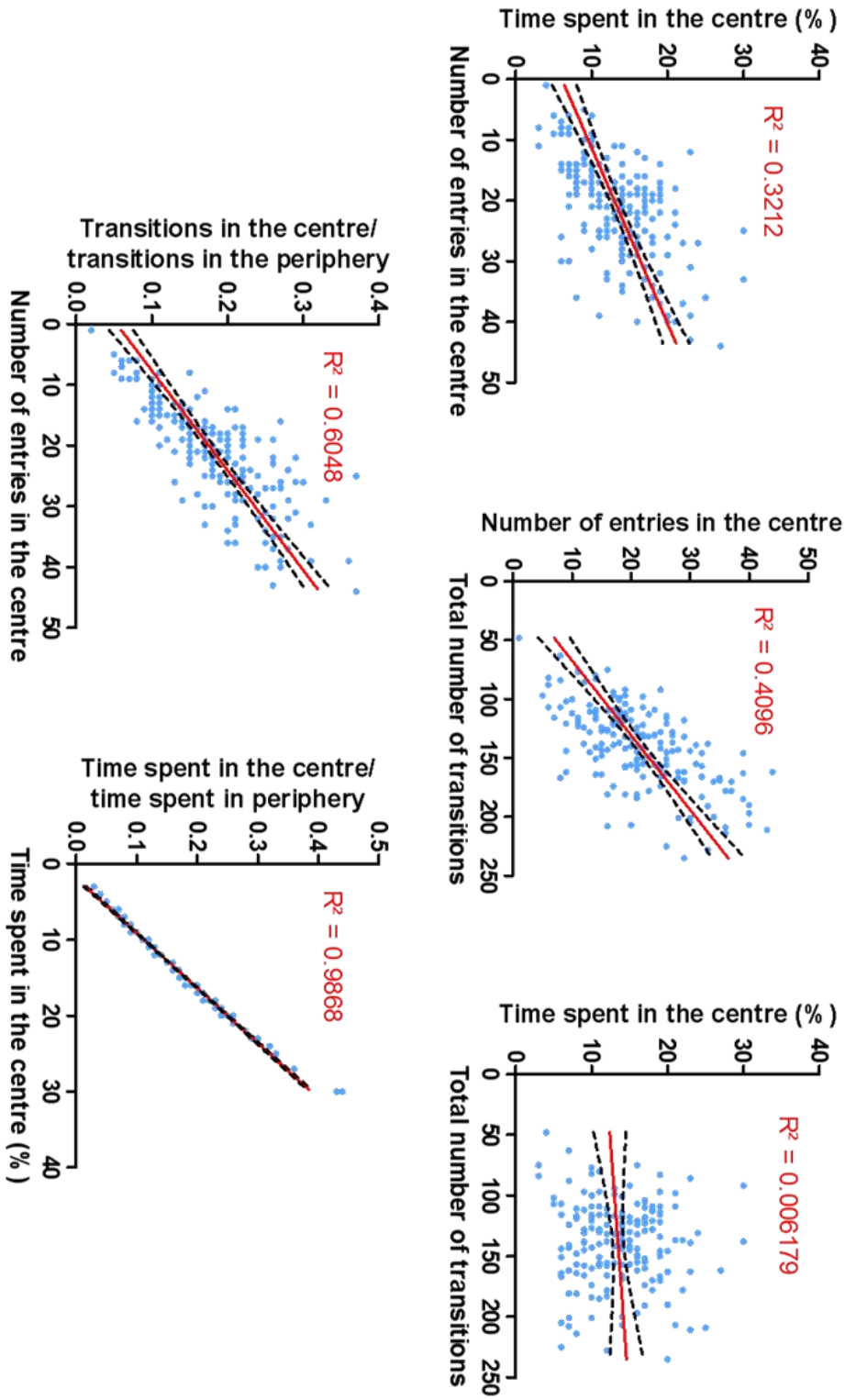
SUPPLEMENTARY DATA 2.1 – Number of mice and normality tests according to the different parameters scored in the open-field.

Bold numbers indicate the mean of the total number of animals per parameter. Statistical codes for Shapiro-Wilk test: *n.s.* $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Number of entries in the centre						
	Batches					
Values	#1	#2	#3	#4	#5	TOTAL
/	/	/	/	/	/	/
0	0	0	0	0	0	0
5	0	0	0	1	0	1
9	1	0	1	4	3	9
13	0	2	4	5	4	15
17	6	3	11	10	6	36
21	**2**	**11**	**7**	**6**	**11**	**37**
26	6	6	8	2	10	32
30	4	8	3	1	7	23
34	4	3	2	2	1	12
38	3	4	0	0	2	9
42	3	1	0	0	2	6
47	0	1	0	0	1	2
Shapiro-Wilk test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Time spent in the centre (%)						
	Batches					
Values	#1	#2	#3	#4	#5	TOTAL
/	/	/	/	/	/	/
1	0	0	0	0	0	0
3	0	0	0	0	0	0
6	1	0	1	3	1	6
8	8	2	5	8	3	26
11	3	2	6	11	9	31
13	**7**	**4**	**5**	**3**	**8**	**27**
16	7	9	9	1	11	37
19	0	7	7	5	8	27
21	2	7	3	0	5	17
24	1	4	0	0	1	6
26	0	2	0	0	0	2
29	0	0	0	0	1	1
Shapiro-Wilk test	n.s.	n.s.	n.s.	**	n.s.	**

Total number of transitions						
	Batches					
Values	#1	#2	#3	#4	#5	TOTAL
37	0	0	0	0	0	0
54	0	0	0	1	0	1
71	0	0	0	0	1	1
88	2	0	3	1	3	9
105	0	3	5	3	2	13
122	3	5	13	7	11	39
139	**7**	**13**	**3**	**3**	**7**	**33**
156	4	5	9	4	9	31
173	3	8	1	8	8	28
190	3	2	1	2	4	12
207	5	0	1	0	2	8
224	1	2	0	1	0	4
241	1	1	0	1	0	3
Shapiro-Wilk test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.



SUPPLEMENTARY DATA 2.2 – Correlations between the different parameters scored.

3 MODULATION OF NEURAL ACTIVITY IN MICE INDUCED BY α-CASOZEPINE

The objective of this study was to evaluate the impact of α-CZP on brain activity in a mouse model. Diazepam was used as a positive control, while the vehicle used to dissolve both molecules was used as a negative control.

Mice were put into two different situations after administration of one molecule or of vehicle: a control situation, where mice were left undisturbed in their housing room, and an anxiety-inducing situation, where mice were put in a light/dark box. Mice were then anesthetised and perfused *in situ* with formol. Brains were harvested, frozen and sliced with a cryostat.

The use of sagittal sections was privileged over coronal sections as it allowed a global investigation of the brain as the precise site of action of α-CZP was still unknown. The use of coronal sections could be used later to precise the action of α-CZP in specific regions.

The nuclear protein c-Fos was used as a marker of neuronal activity, its expression being evaluated with immunofluorescence. The density of c-Fos positive neurons in a specific region was used as a evaluation of the activity of this area. The use of mouse brain atlas, allowed the identifications of several regions involved in anxiety regulation: amygdala, BNST, hippocampal formation, hypothalamus, prefrontal cortex, accumbens nucleus.

The results of this study have been published in *Journal of Functional Foods*:

Benoit, S., Chaumontet, C., Schwarz, J., Cakir-Kiefer, C., Tomé, D., Miclo L, 2017. Mapping in mice the brain regions involved in the anxiolytic-like properties of α-casozepine, a tryptic peptide derived from bovine αs1-casein. Journal of Functional Foods. 38: 464-473.

In summation

- An anxiety-inducing situation is mandatory to trigger the anxiolytic-like properties of α-CZP.
- This is one of the first time that a bioactive peptide has been shown to trigger modulation of neuronal activity in the brain after an i.p. injection in a mouse model.
- The modulation of neuronal activity induced by α-CZP is different from that of diazepam:
    - α-CZP had no impact on the activity of the prefrontal cortex while diazepam decreased it.
    - α-CZP increased the activity of the amygdala while diazepam had no effect on it.

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Mapping in mice the brain regions involved in the anxiolytic-like properties of α-casozepine, a tryptic peptide derived from bovine α_{s1}-casein

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ABSTRACT

α-Casozepine, a bioactive peptide from milk casein, displays an anxiolytic-like activity in many species. Since its mode of action is still not elucidated, a study was conducted in Swiss mice to investigate c-Fos expression, a marker of neuronal activity, in different brain areas. After an intraperitoneal injection of α-casozepine (1 mg/kg), animals were placed either in a non-stressful or in an anxiety-inducing situation triggered with a light/dark box. No effect of α-casozepine on c-Fos expression was observed in the non-stressful situation. In the stressful situation, modulation of neuronal activity by α-casozepine was observed in different brain regions compared to that of vehicle. However, while diazepam, a benzodiazepine, modulated neuronal activity the same way in hippocampus, accumbens nucleus and hypothalamus, differences were observed in c-Fos expression in amygdala and prefrontal cortex compared to α-casozepine. These results strengthen the assumption that the anxiolytic mechanisms of α-casozepine differ partly of those of diazepam.

1. Introduction

Amongst food protein-derived bioactive peptides targeting physiological systems (Udenigwe & Aluko, 2012), a tryptic hydrolysate of bovine α_{s1}-casein (CH) exerts anticonvulsant and anxiolytic properties when injected by intraperitoneal (i.p.) way to rats (Miclo et al., 2001) and displays anxiolytic-like profile when orally administered to rats (Violle et al., 2006), dogs (Beata, Beaumont-Graff, Diaz, et al., 2007; Palestrini et al., 2010), cats (Beata, Beaumont-Graff, Coll, et al., 2007), horses (McDonnell, Miller, & Vaala, 2014) and humans (Kim et al., 2007; Messaoudi, Lefranc-Millot, Desor, Demagny, & Bourdon, 2005). It also demonstrated sleep-modulating effects in rodents (Dela Peña et al., 2016; Guesdon et al., 2006). The industrially produced CH received health claims from national agencies (Australia, France, South Korea, United States) and is currently used in both human and veterinary medicines. In this hydrolysate, the decapeptide α-casozepine (α-CZP, YLGYLEQLLR, residues 91–100 of the mature chain of bovine α_{s1}-casein) was identified as potential carrier of the anxiolytic-like activity (Miclo et al., 2001).

Different properties of α-CZP lead to consider this peptide as a benzodiazepine (BZD)-like molecule, the most prescribed drug family to cure anxiety disorders: (i) the distance between the centres of the two tyrosine aromatic rings of the peptide in a micellar medium was similar with that of the aromatic rings of the BZD nitrazepam (Lecouvey et al., 1997); (ii) the peptide displayed an affinity for the BZD site of the $GABA_A$ receptor (but 10,000 times lower than that of diazepam) (Miclo et al., 2001); (iii) CH induced a $GABA_A$ receptors-mediated increase of Cl^- influx in neuroblastoma cell culture (Dela Peña et al., 2016). In contrast, other results suggest a distinct mode of action between α-CZP and diazepam: (i)

Abbreviations: α-CZP, α-casozepine; AIS, anxiety-inducing situation; BNST, bed nucleus of the stria terminalis; BZD, benzodiazepine; CH, tryptic hydrolysate of bovine α_{s1}-casein; CS, control situation; GABA, γ-amino butyric acid; i.p., intraperitoneal; LDB, light/dark box.

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1756-4646/

compared to diazepam, CH induced neither habituation, dependence, sedation, memory impairment nor toxicity (Dela Peña et al., 2016; Messaoudi, Lalonde, Schroeder, & Desor, 2009); (ii) CH may only exert its anxiolytic-like properties in stressed situation or amongst stressed populations as demonstrated by improvement of either sleep after chronic mild stress in rats (Guesdon et al., 2006), stress-related symptoms in stressed women (Kim et al., 2007), or anxiety in anxious Beagle dogs (Palestrini et al., 2010).

Despite these numerous studies, the mode of action of the peptide is still not clarified. As some gathering evidences point out a central mode of action (Dela Peña et al., 2016; Guesdon et al., 2006), the goal of this study was to evaluate the impact of a single i.p. injection of α-CZP compared to diazepam on neuronal activity, by evaluating c-Fos expression, a neuronal activity marker (Chung, 2015; Kovács, 1998), both in a control and an anxiety-inducting (*via* a light/dark box device) situations.

2. Material and methods

2.1. Drugs and molecules

α-CZP (1 mg/kg, Genosphere Biotechnologies, France) and diazepam (1 mg/kg, Valium, Roche, Switzerland) were respectively diluted or suspended in a 1% (v/v) glycerol, 0.2% (w/v) methylcellulose aqueous solution (vehicle). The doses used for the two compounds were evaluated in a preliminary study based on data from rodents (unpublished results).

2.2. Animals

All experiences were approved by the French « Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt » on the recommendation of the Comité d'Éthique en Expérimentation Animale of Jouy-en-Josas (N°02237.01). Male Swiss mice ($n = 48$) (Janvier Labs, France), aged of 9 weeks at arrival, were housed individually and maintained on a 12 h inversed light/dark cycle (lights off at 08:00 a.m.) and controlled environment (temperature 22 ± 1 °C, humidity 60%). Water and food were available *ad libitum*. Mice were handled every day by the same experimenter and particular care was taken to limit any kinds of external stress (variations in light, noise or odour). Behavioural tests were performed during their active period.

2.3. Light/dark box (LDB) test

The device was inspired by Crawley and Goodwin's previous work (Crawley & Goodwin, 1980). This homemade apparatus consisted in two connected boxes (12 × 16 × 20 cm and 24 × 16 × 20 cm). The smaller box was darkened. A light from a 60 W (1000 lux) desk lamp provided the only room illumination. The animals were individually tested in 5-min sessions. Mice were placed in the lit box facing the entrance to the dark box to start the test session. Behaviour was recorded using a camera (Logitech, Switzerland), while the experimenter was waiting outside of the room in order to not disturb the behaviour of the mice. The latency to enter the dark, unknown box, the total number of transitions between the two compartments, the time spent in the lit box and the number of rears in the lit box were recorded under blinded conditions using The Observer XT 8.0 (Noldus, The Netherlands).

To firstly assess the anxiolytic-like properties of α-CZP in the LDB model, animals ($n = 8$/group) were placed in the LDB, thirty minutes after an i.p. injection (5 mL/kg body weight) of either vehicle, α-CZP (1 mg/kg corresponding to 0.8 μmol/kg) or diazepam (1 mg/kg corresponding to 3.5 μmol/kg). Behaviours were recorded for five minutes and analysed before the beginning of the experimental procedure described below.

2.4. Experimental procedure: control and anxiety-inducing situations

After a week of acclimation to the housing conditions, animals received a soy protein-based diet (standard AIN-93M diet containing 20% of total energy as soy protein, 10% as fat and 70% as carbohydrate) in order to exclude a potential endogenous casein-derived formation of α-CZP (Fig. 1A). Two weeks later, animals ($n = 8$/group) received an i.p. injection of 5 mL/kg body weight of either vehicle, α-CZP (1 mg/kg) or diazepam (1 mg/kg). Half of the animals in each treatment group ($n = 4$/subgroup) were injected and returned to their housing room after injection this approach was called "control situation" (CS; Fig. 1B). The other half ($n = 4$/subgroup) was placed back to its housing room for 30 min and subsequently in the LDB apparatus for 5 min leading mice to an anxiety-inducing situation (AIS; Fig. 1C). LDB was used as a stressor and not as an apparatus to evaluate anxiety. They were returned to their housing room after the 5-min session. The experiments were performed during six days divided in two weeks, during morning sessions (the behavioural tests were executed between 8:30 and 11:00 a.m.). In each session, six animals (one from each experimental group) were euthanized. All animal transfers as well as injections were performed in the dark or under a dim red light. All the animals have undergone exactly the same handlings with exception of the stressful situation.

2.5. Brain collection and sectioning

Protocol was inspired from past work (Schwarz et al., 2010). Two hours after the injection (Fig. 1B and C), mice received an i.p. injection of sodium pentobarbital (100 mg/kg, Ceva Santé Animale, France). They were subsequently transcardially perfused via a 21G needle (inner diameter: 0.514 mm) placed in the left cardiac ventricle with 50 mL of Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 0.05% (w/v) $NaNO_2$, followed by 100 mL of 4% (v/v) formaldehyde (Microm Microtech, France). Brains were afterwards harvested and post-fixed for 72 h in 4% (v/v) formaldehyde at 4 °C. Thereafter, for cryo-protection, brains were immersed for 24 h in 15% (w/v) sucrose (Alfa Aesar, Germany) solution at 4 °C and subsequently in 30% sucrose solution at 4 °C during 48 h. The brains were then snap-frozen at −80°C embedded in Tissue-Tek® (Sakura Finetek Europe, The Netherlands) and stored at −20°C until sectioning. The sagittal 20 μm-thick slices were sampled every 100 μm, covering the entire left hemisphere (approximately 25 slices per animal), using a CM1520 cryostat (Leica, Germany).

2.6. c-Fos immunofluorescence

Slices were rehydrated three times in a Phosphate-Buffered Saline (PBS) solution during 10 min. They were then incubated in the same PBS solution containing 0.5% (v/v) Triton X-100 and 2% (w/v) BSA during an hour at room temperature. Afterwards they were incubated in a new PBS/Triton X-100/BSA solution containing in addition 1.5% goat serum and rabbit anti-c-Fos antibody (primary antibody, 1:5000, Ab-5, Calbiochem, France) during 48 h at 4 °C. Slices were next rinsed three times 10 min in PBS solution and then incubated in the PBS/Triton X-100/BSA solution containing Alexa-Fluor 488 (secondary antibody, 1:200, Molecular Probes, France) for 2 h at room temperature. Eventually, slices were washed three times in PBS solution and mounted using a medium containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, France). Two c-Fos negative controls, obtained by omitting either the primary or the secondary antibody, were processed during each

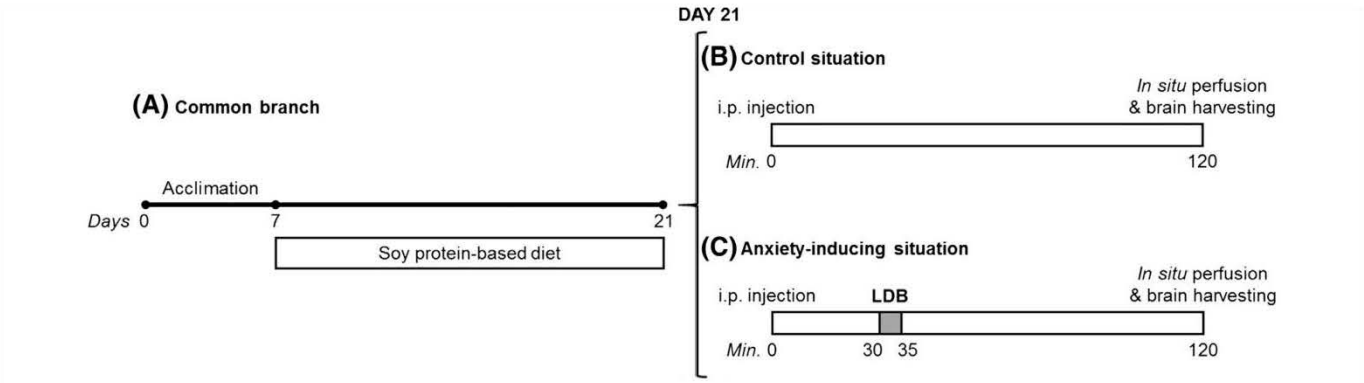

Fig. 1. Schematic representation of the experimental design. (A) Common branch of the experiment: after a week of acclimation to the housing conditions, mice were given a soy protein diet *ad libitum*. Two weeks later, mice were randomised into two different situations. (B) Control situation: after an i.p. injection (vehicle, α-CZP or diazepam), mice were placed back in their housing room for 120 min before *in situ* perfusion. (C) Anxiety-inducing situation: 30 min after an i.p. injection (vehicle, α-CZP or diazepam), mice were placed in the light/dark box (LDB) device for 5 min; 90 min later mice, were perfused *in situ*.

experiment to check for negative controls. No staining was observed on these two controls (data not shown).

2.7. Automatic c-Fos positive cell counting

Sagittal slices were digitised at INSERM UMR S996 (Paris, France) using a Nanozoomer (Hamamatsu, Japan) equipped with a ×20 objective lens and epifluorescence. Different image optimisation steps were thereafter performed on ImageJ v1.49 (Rasband, 1997) to facilitate the automated counting. First, the green channel was extracted and pictures were afterwards inversed. An automated counting was performed in a given region using the *Find maxima* function of ImageJ. This method was repeatedly attested with manual counting control on a non-transformed image in the central amygdaloid nucleus (Fig. 2). The automated cell counting method we optimised showed a strong correlation with the manual counts ($y = 1.09x - 1.7$, $R^2 = 0.9809$). As this method was confirmed in our protocol, it allowed us to have a more objective counting method. The number of c-Fos positive cell nuclei within each brain region was counted in minimum six sections per animal and the average of them was expressed as number of c-Fos neurons/0.04 mm^2. Investigated brain regions were upstream selected due to their implication in anxiety regulation (Calhoon & Tye, 2015; Noori, Spanagel, & Hansson, 2012). All

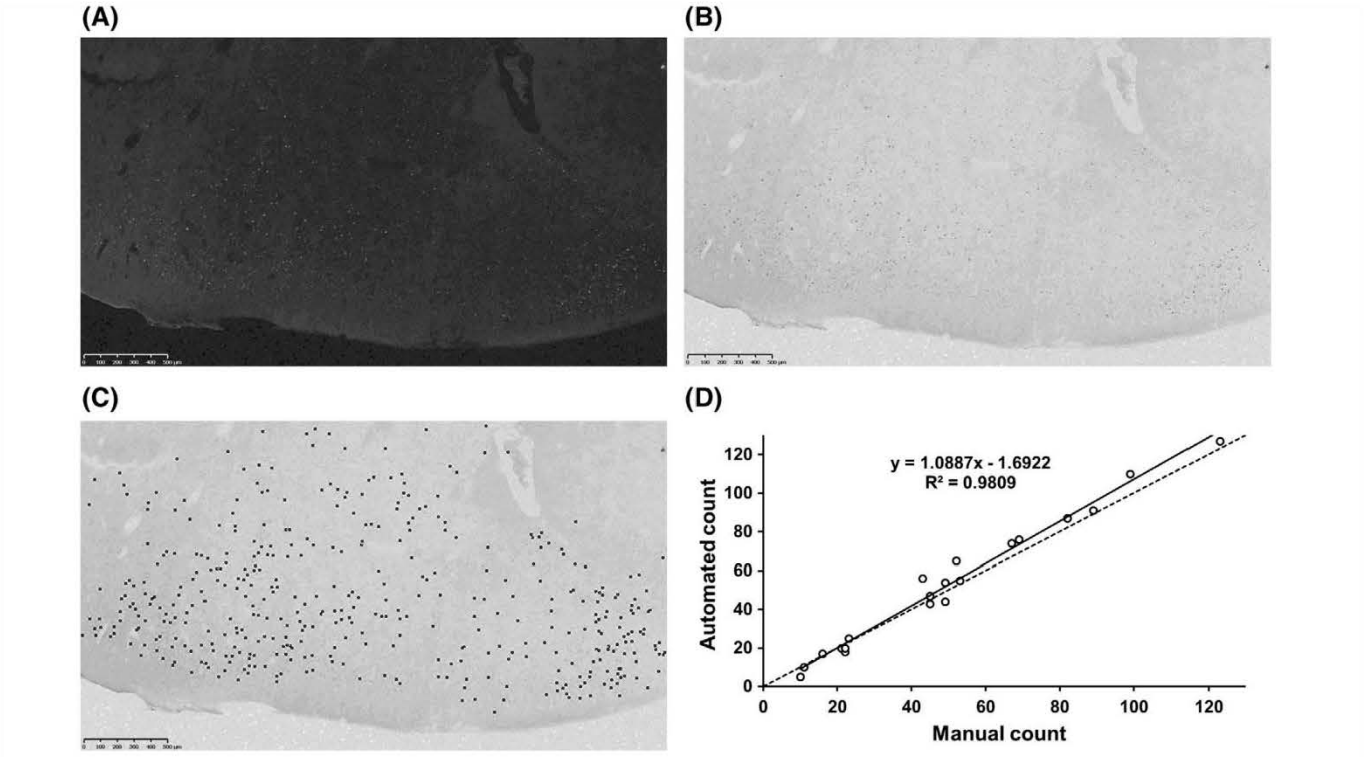

Fig. 2. Procedure used for the c-Fos positive neurons automated count. (A) Zoom on the amygdala region on a digitalised slice (position: lateral 2.52 mm). Extraction of the green canal. Scale bar: 500 μm. (B) The same slice after colour inversion. Scale bar: 500 μm. (C) Automated counting using the Find maxima function of ImageJ on the inversed image. Black dots correspond to the c-Fos positive neurons counted by the software. Scale bar: 500 μm. (D) Automated cell counts plotted against manual cell counts. Cells were counted on 20 different slices in the central amygdaloid nucleus. The regression line (solid line, $y = 1.09x - 1.7$, $R^2 = 0.9809$) was close to $y = x$ (broken line).

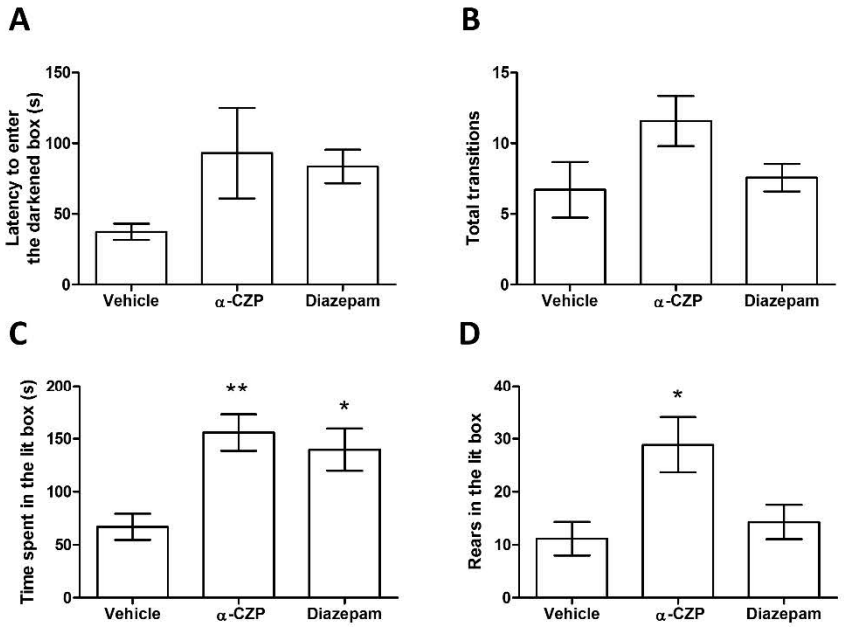

Fig. 3. Effects of an i.p. injection of α-CZP (1 mg/kg), and diazepam (1 mg/kg) in Swiss mice on the behavioural response in the light/dark box. Results were analysed using a one-way ANOVA to detect the effect of the treatment on the studied behavioural scores. Data are mean ± SEM (n = 7–8/group). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the vehicle.

regions were assessed using a stereotaxic atlas ((Paxinos & Franklin, 2001); Figs. 4 and 5).

2.8. Statistics

Data were evaluated with one- or two-way analysis of variance (ANOVA), after assessing the normal distribution of the residuals. Multiple comparison analysis was performed with Bonferroni post hoc tests using R (R Core Team, 2013). All data are reported as mean ± SEM. Differences were considered to be significant at the $p < 0.05$ level.

3. Results

3.1. Anxiolytic-like properties of α-CZP in mice in the LDB

The impact of i.p. injection of α-CZP on the behaviour of mice in an anxiogenic situation was evaluated using the LDB (Fig. 3). A-CZP compared to the vehicle increased the time spent in the lit box (×2.3, $F_{(2,18)} = 8.0112$, $p = 0.0043$) as well as the number of rears in the lit box (×2.6, $F_{(2,18)} = 5.608$, $p = 0.0170$). α-CZP had no effect on the latency to enter the unknown dark compartment and on total transitions. Diazepam only increased the time spent in the lit box compared to the vehicle (×2.3, $F_{(2,18)} = 8.0112$, $p = 0.0196$).

3.2. c-Fos expression in the neural circuits of anxiety in vehicle-treated animals facing CS or AIS

c-Fos expression was measured in animals belonging to the vehicle-treated group and compared between CS and AIS (Table 1). All regions studied in the animals facing CS displayed a scarce distribution of c-Fos positive neurons (min: 0.85 cells/0.04 mm^2 in the caudate putamen – max: 4.32 cells/0.04 mm^2 in the dorsomedial nucleus of the hypothalamus), compared to those of animals facing AIS (min: 1.49 cells/0.04 mm^2 in the central nucleus of the amygdala – max: 18.75 cells/0.04 mm^2 in the ventromedial nucleus of the hypothalamus).

3.2.1. Prefrontal cortex

The AIS had an overall effect on c-Fos positive neurons in the prefrontal cortex, significantly increasing the number of these neurons compared to what was observed with the CS (×3.6, $F_{(1,18)} = 109.0087$, $p < 0.0001$).

3.2.2. Hippocampal formation

The AIS had a global influence on c-Fos positive neurons in the hippocampal formation, significantly increasing the number of these neurons compared to what was counted with the CS (×5.4, $F_{(1,18)} = 111.287$, $p < 0.0001$).

3.2.3. Accumbens nucleus

The AIS had an over-all effect on c-Fos positive neurons in the accumbens nucleus, significantly increasing the number of these neurons compared to what was observed with the CS (×2.1, $F_{(1,18)} = 17.9776$, $p = 0.0003$), but this increase was only significant in the core (×2.6, $F_{(1,18)} = 36.8749$, $p < 0.0001$).

3.2.4. Hypothalamus

The AIS compared to the CS had no effect on c-Fos positive neurons in the overall hypothalamic structure. However, the number of c-Fos positive neurons was significantly increased in some nuclei i.e. the dorsomedial (×3.8, $F_{(1,18)} = 52.997$, $p < 0.0001$), the paraventricular (×4.4, $F_{(1,18)} = 476.173$, $p < 0.0001$) and the ventromedial (×5.2, $F_{(1,18)} = 57.615$, $p < 0.0001$).

3.2.5. Amygdala

Compared to the CS, the AIS had no influence on c-Fos positive neurons in the overall amygdala, nor in the different sub-regions.

3.2.6. Other regions

The AIS increased the number of c-Fos positive neurons compared to what was noticed with the CS in the septum (×3.7, $F_{(1,18)} = 31.4659$, $p = 0.0398$) and the BNST (×5.4, $F_{(1,18)} = 129.5467$, $p < 0.0001$), but no effect was observed in the caudate putamen compared to the CS.

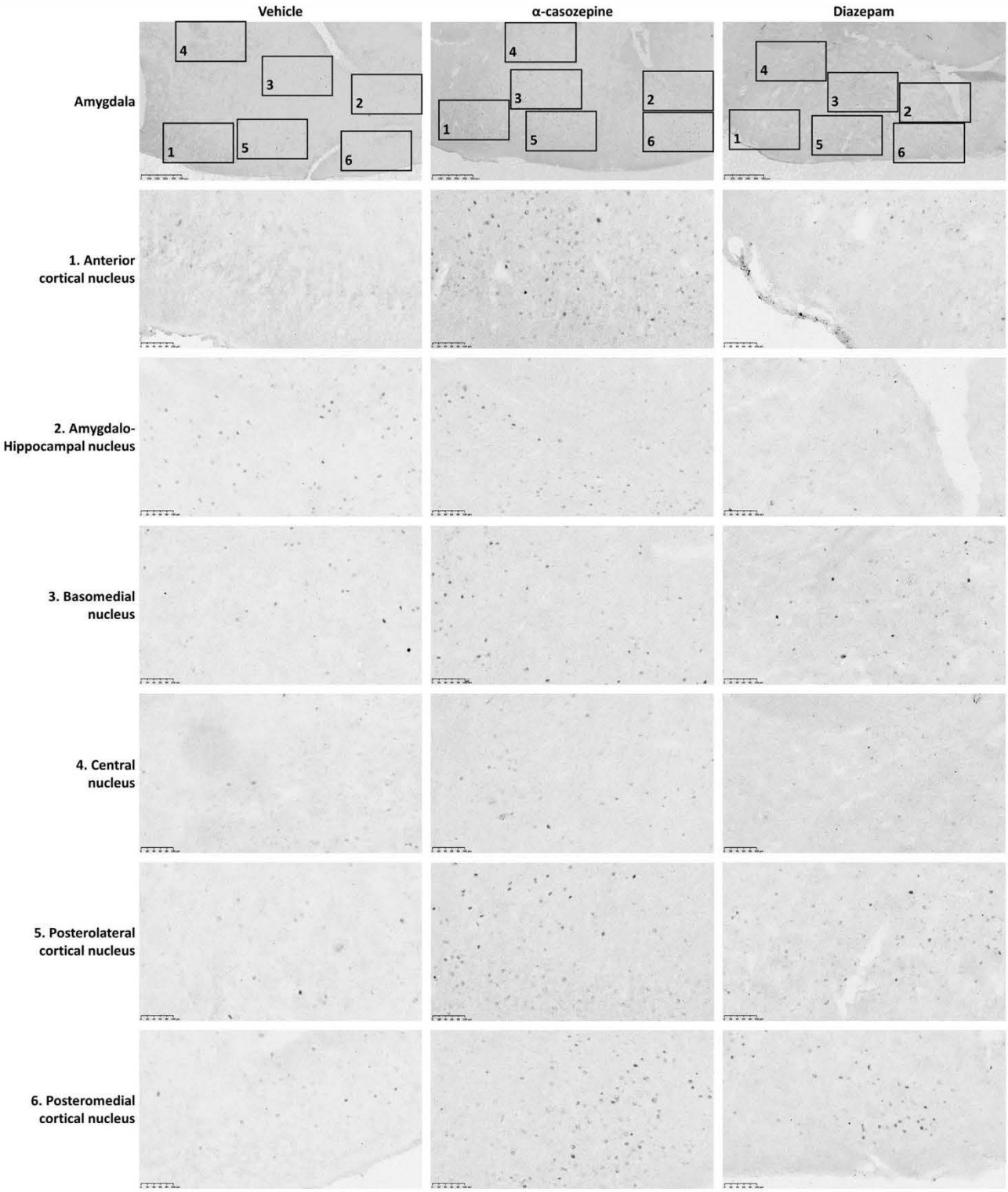

Fig. 4. Photomicrographs illustrating c-Fos immunoreactivity in the amygdala (lateral 2.52 mm) of mice administrated with vehicle (left), α CZP (1 mg/kg i.p.; middle), and diazepam (1 mg/kg i.p.; right) before facing an anxiety-inducing situation. The green channel was extracted and the images were inversed. Contrast was increased to 40% in the images from line 2 to line 7. Global amygdala in first line, scale bar: 500 μm. Regions of amygdala in the following lines, scale bar: 100 μm.

3.3. c-Fos expression in the neural circuits of anxiety in α-CZP and diazepam-treated animals facing CS or AIS

c-Fos expression was measured in animals belonging to the α-CZP and diazepam-treated group facing CS or AIS (Table 1). In the CS, compared to an injection of vehicle, no effect of an i.p. injection of α-CZP was observed in the regions listed above on c-Fos positive neurons. The i.p. injection of diazepam, on the other hand, decreased the number of c-Fos positive neurons globally in the accumbens nucleus ($\times 0.4$, $F_{(2,18)} = 35.6236$, $p = 0.0395$) and

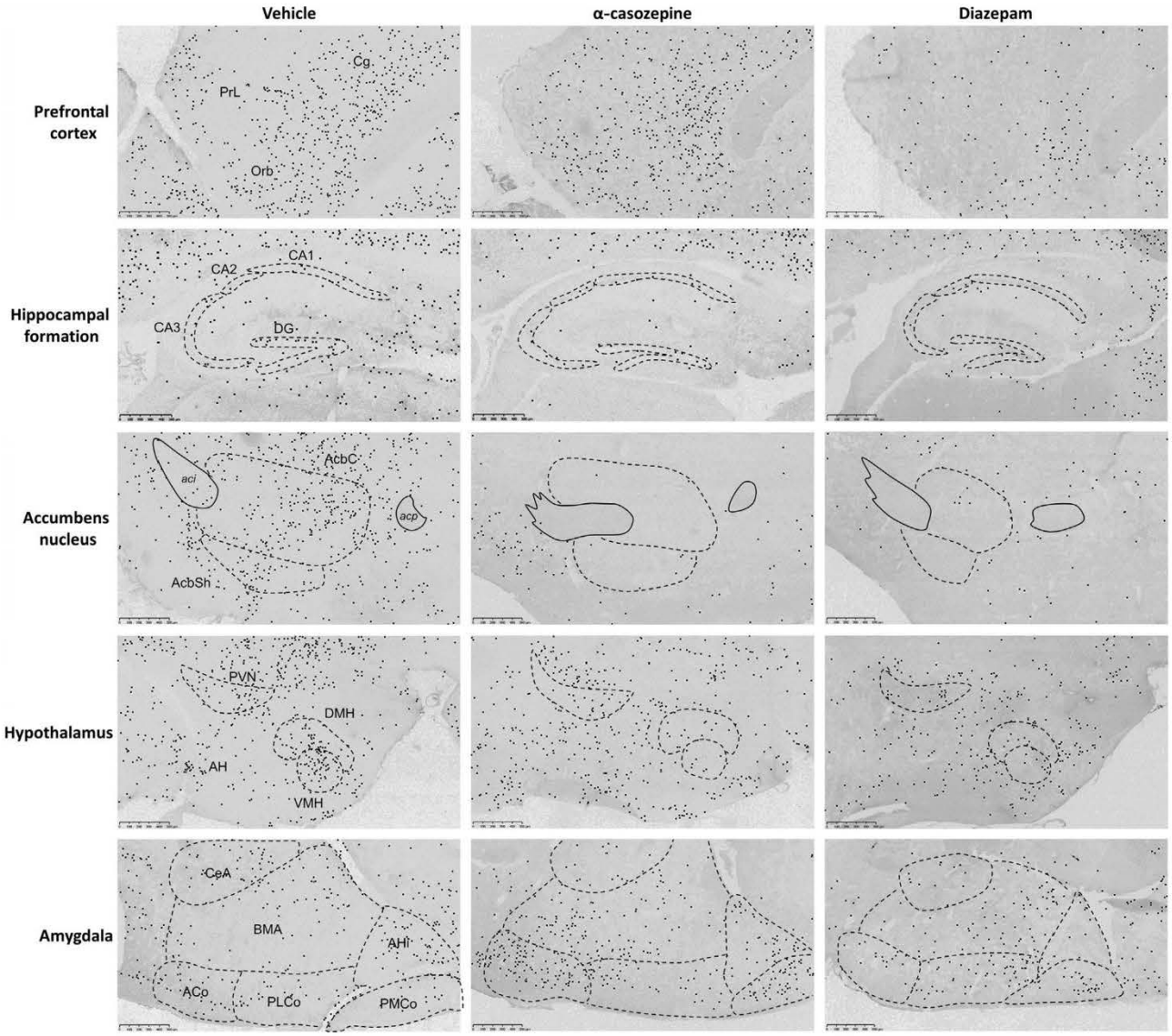

Fig. 5. Photomicrographs illustrating the effect of the vehicle, α-CZP (1 mg/kg, i.p.), and diazepam (1 mg/kg, i.p.) on anxiety-induced c-Fos immunoreactivity in the prefrontal cortex (lateral 0.12 mm), hippocampal formation (lateral 0.48 mm), accumbens nucleus (lateral 0.84 mm), hypothalamus (lateral 0.12 mm) and amygdala (lateral 2.52 mm). Images followed the procedure as described in Fig. 2: green channel was extracted and images were inversed; black dots correspond to the c-Fos positive neurons extracted by ImageJ, using the *Find Maxima* function. Scale bar: 500 μm. Cg: cingulate cortex; PrL: prelimbic cortex; Orb: orbital cortex; CA1: field CA1 of hippocampus; CA2: field CA2 of hippocampus; CA3: field CA3 of hippocampus; DG: dentate gyrus; AcbC: accumbens nucleus, core; AcbSh: accumbens nucleus, shell; aci: anterior commissure, intrabulbar; acp: anterior commissure, posterior; AH: anterior hypothalamic nucleus; DMH: dorsomedial hypothalamic nucleus; PVN: paraventricular hypothalamic nucleus; VMH: ventromedial hypothalamus nucleus; Aco: anterior cortical amygdaloid nucleus; AHi: amygdalohippocampal area; BMA: basomedial amygdaloid nucleus; CeA: central amygdaloid nucleus; PLCo: postlateral cortical amygdaloid nucleus; PMCo: posteromedial cortical amygdaloid nucleus.

specifically in the shell (×0.3, $F_{(2,18)} = 29.2793$, $p = 0.0146$) as well as in the paraventricular nucleus of the hypothalamus (×0.3, $F_{(2,18)} = 43.780$, $p = 0.0318$) compared to the vehicle.

In contrast, effects of an i.p. injection of α-CZP and diazepam could be observed in some regions in the AIS (Table 1 and Figs. 4 and 5).

3.3.1. Prefrontal cortex

No effect of α-CZP was observed in the prefrontal cortex globally on the number of c-Fos positive neurons compared to what was noted with the vehicle. Nevertheless, a significant decrease of this number was observed in the frontal association cortex (×0.4, $F_{(2,18)} = 19.468$, $p = 0.0146$). On the other hand, diazepam decreased the number of c-Fos positive neurons in all the regions of the prefrontal cortex globally compared to the vehicle. The only difference between effects of α-CZP and diazepam was observed in the cingulate cortex ($F_{(2,18)} = 21.3798$, $p = 0.0001$).

3.3.2. Hippocampal formation

α-CZP decreased the number of c-Fos positive neurons globally in the hippocampal formation (×0.6, $F_{(2,18)} = 23.884$, $p = 0.0066$) compared to the vehicle, and more specifically in the CA2 (×0.4, $F_{(2,18)} = 25.123$, $p = 0.0002$) and in the dentate gyrus (×0.5, $F_{(2,18)} = 25.433$, $p = 0.0002$). On the other hand, diazepam decreased the number of c-Fos positive neurons in the hippocampal formation globally (×0.3, $F_{(2,18)} = 23.884$, $p < 0.0001$) compared to the vehicle. A difference between effects of α-CZP and diazepam was observed globally in the hippocampal formation ($F_{(2,18)} = 23.884$, $p = 0.0092$) and more specifically in the CA1 ($F_{(2,18)} = 21.485$, $p < 0.0001$).

Table 1
The effects of an i.p. injection of α-CZP (1 mg/kg) or diazepam (1 mg/kg) on the anxiety-induced c-Fos immunoreactivity (positive cells/0.04 mm^2) in different areas of mice brains ($n = 4$/group). Data are mean ± SEM. Results were analysed using a two-way ANOVA (light/dark box × treatment) to detect the effects of an anxiety-inducing situation (A) and treatment (T) and the interaction (A * T) of these factors ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Subsequently, a Bonferroni post hoc test was performed to compare the effect of the anxiety-inducing situation and the effect of α-CZP and diazepam compared to vehicle within each situation: a, significantly different from the Vehicle/CS group; b, significantly different from the Vehicle/AIS group; c, significantly different between α-CZP/AIS and DZP/AIS groups ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Brain region	Control Situation (CS)			Anxiety-Inducing Situation (AIS)			ANOVA					
	Vehicle	α-CZP	Diazepam	Vehicle	α-CZP	Diazepam	A		T		A * T	
							$F_{(1,18)}$	p	$F_{(2,18)}$	p	$F_{(2,18)}$	p
Prefrontal cortices												
Global	2.11 ± 0.40	1.72 ± 0.24	0.72 ± 0.26	7.67 ± 0.42, a***	5.73 ± 0.86	3.89 ± 0.53, b***	109.0087	***	13.5196	***	2.9927	*ns*
Cingulate cortex	2.50 ± 0.40	1.93 ± 0.23	0.90 ± 0.34	9.92 ± 0.39, a***	9.81 ± 1.26, c***	3.57 ± 0.82, b***	118.3641	***	21.3798	***	9.1502	**
Frontal association cortex	2.44 ± 0.19	2.02 ± 0.39	1.05 ± 0.49	11.28 ± 1.41, a***	4.25 ± 0.83, b***	3.99 ± 0.55, b***	57.703	***	19.468	***	11.663	***
Prelimbic cortex	1.86 ± 0.40	1.62 ± 0.40	0.61 ± 0.31	6.75 ± 0.96, a***	4.60 ± 0.50	3.16 ± 0.76, b**	50.2738	***	8.1627	**	2.1766	*ns*
Orbital cortex	2.13 ± 0.48	1.65 ± 0.22	0.68 ± 0.22	7.33 ± 0.59, a***	5.31 ± 0.91	4.23 ± 0.59, b*	83.4489	***	8.4173	**	1.3760	*ns*
Hippocampal formation												
Global	1.14 ± 0.18	1.04 ± 0.13	0.45 ± 0.08	6.16 ± 0.64, a***	3.96 ± 0.50, b**, c***	1.85 ± 0.27, b***	111.287	***	23.884	***	12.684	***
CA1	0.89 ± 0.22	0.77 ± 0.20	0.40 ± 0.10	4.05 ± 0.33, a***	4.14 ± 0.60, c***	0.91 ± 0.18, b***	81.377	***	21.485	***	12.628	***
CA2	1.51 ± 0.27	1.81 ± 0.26	0.70 ± 0.11	5.84 ± 0.72, a***	2.63 ± 0.35, b***	1.25 ± 0.28, b***	37.193	***	25.123	***	15.372	***
CA3	1.29 ± 0.16	1.05 ± 0.19	0.45 ± 0.11	5.90 ± 1.10, a***	4.04 ± 0.76	2.06 ± 0.27, b**	44.0876	***	8.5467	**	3.5008	*ns*
Dentate gyrus	1.47 ± 0.36	1.27 ± 0.16	0.46 ± 0.09	8.32 ± 0.80, a***	4.29 ± 0.69, b***	2.50 ± 0.35, b***	101.229	***	25.433	***	13.836	***
Accumbens nucleus												
Global	2.22 ± 0.16	2.09 ± 0.40	0.80 ± 0.13, a*	4.58 ± 0.37, a***	2.39 ± 0.37, b***	1.13 ± 0.17, b***	17.9776	***	35.6236	***	8.3374	***
Core	2.05 ± 0.18	2.13 ± 0.39	0.77 ± 0.19	5.42 ± 0.41, a***	3.19 ± 0.52, b**	1.54 ± 0.26, b***	36.8749	***	27.5811	***	8.2868	**
Shell	2.58 ± 0.17	2.31 ± 0.54	0.88 ± 0.16, a*	3.82 ± 0.34	1.70 ± 0.29, b*	0.85 ± 0.10, b***	0.6746	*ns*	29.2793	***	4.8089	***
Hypothalamus												
Global	2.56 ± 0.13	2.43 ± 0.36	1.97 ± 0.20	4.85 ± 0.43	5.58 ± 1.05	3.24 ± 0.58	24.8310	***	3.5982	*	1.4455	*ns*
Anterior area	2.09 ± 0.18	2.12 ± 0.49	2.67 ± 0.29	5.12 ± 0.46	5.14 ± 1.37	3.75 ± 0.63	17.9134	***	0.2351	*ns*	1.3269	*ns*
Dorsomedial nucleus	4.32 ± 0.67	3.21 ± 0.38	1.27 ± 0.16	16.62 ± 1.56, a***	4.82 ± 1.63, b***	5.96 ± 0.90, b***	52.997	***	27.226	***	13.910	***
Lateral area	2.87 ± 0.34	2.50 ± 0.26	1.74 ± 0.34	3.46 ± 0.34	2.38 ± 0.64	1.93 ± 0.46	0.4148	*ns*	5.1283	*	0.3682	*ns*
Paraventricular nucleus	4.17 ± 0.54	3.95 ± 0.27	1.25 ± 0.17, a*	18.41 ± 0.48, a***	10.89 ± 0.15, b***	10.93 ± 1.16, b***	476.173	***	43.780	***	20.376	***
Ventromedial nucleus	3.61 ± 0.42	3.38 ± 0.23	1.20 ± 0.14	18.75 ± 1.85, a***	5.41 ± 1.57, b***	6.69 ± 1.67, b***	57.615	***	22.149	***	15.534	***
Amygdala												
Global	1.24 ± 0.13	1.16 ± 0.07	1.22 ± 0.09	2.80 ± 0.63	7.95 ± 1.23, b***, c***	4.33 ± 0.90	47.9560	***	7.4363	**	7.8751	**
Anterior cortical nucleus	1.27 ± 0.13	1.53 ± 0.24	1.50 ± 0.31	3.29 ± 0.85	5.51 ± 0.79, c***	2.66 ± 0.49	29.3636	***	4.1712	*	3.5805	*
Amygdalohippocampal nucleus	1.28 ± 0.13	1.27 ± 0.19	1.16 ± 0.25	2.29 ± 0.34	8.40 ± 0.94, b***, c***	2.80 ± 0.73	57.727	***	21.134	***	20.616	***
Basolateral nucleus	1.26 ± 0.19	0.94 ± 0.18	0.88 ± 0.22	1.78 ± 0.25	7.85 ± 0.77, b***, c***	4.03 ± 0.94	67.587	***	15.519	***	18.662	***
Basomedial nucleus	0.97 ± 0.21	1.23 ± 0.24	1.15 ± 0.36	3.43 ± 0.89	5.39 ± 0.94	2.73 ± 0.54	30.5536	***	2.8715	*ns*	2.3217	*ns*
Central nucleus	1.45 ± 0.23	1.21 ± 0.19	1.19 ± 0.25	1.68 ± 0.47	3.68 ± 0.51	3.98 ± 1.16	15.1802	**	1.8538	*ns*	2.9585	*ns*
Medial nucleus	1.47 ± 0.19	1.60 ± 0.37	1.43 ± 0.24	2.33 ± 0.55	13.53 ± 2.87, b***, c***	7.55 ± 1.27	34.5245	***	9.2979	***	8.8684	**
Posterolateral cortical nucleus	1.20 ± 0.18	1.42 ± 0.18	1.36 ± 0.34	4.75 ± 1.92	5.22 ± 0.86	1.96 ± 0.50	13.0093	**	1.8918	*ns*	1.9710	*ns*
Posteromedial cortical nucleus	1.27 ± 0.14	1.34 ± 0.16	1.23 ± 0.26	3.70 ± 0.70	9.41 ± 1.20, b***, c***	3.32 ± 0.59	66.564	***	15.136	***	14.206	***
Other regions												
Septum	1.08 ± 0.19	1.21 ± 0.28	1.29 ± 0.18	3.93 ± 0.29, a*	6.04 ± 1.30, c***	1.56 ± 0.30	31.4659	***	7.2480	**	7.8071	**
Bed nucleus of the stria terminalis	0.91 ± 0.24	0.91 ± 0.21	1.24 ± 0.14	4.92 ± 0.47, a***	2.84 ± 0.21, b**	3.57 ± 0.37	129.5467	***	6.1655	**	6.9068	**
Caudate putamen	0.85 ± 0.42	0.90 ± 0.20	0.90 ± 0.18	1.49 ± 0.19	2.12 ± 0.40, c***	0.63 ± 0.08	5.6923	*	3.7003	*	3.7279	*

3.3.3. Accumbens nucleus

Both i.p. injection of α-CZP or diazepam decreased the number of c-Fos positive neurons globally in the accumbens nucleus (×0.5, $F_{(2,18)} = 35.6236$, $p = 0.0006$ and ×0.2, $F_{(2,18)} = 35.6236$, $p < 0.0001$, respectively) compared to the vehicle. No difference was observed between α-CZP and diazepam.

3.3.4. Hypothalamus

No effect of both i.p. injection of α-CZP and diazepam compared to that of vehicle was observed globally in the hypothalamus, as well as in the anterior and lateral nuclei. However, both α-CZP and diazepam decreased the number of c-Fos positive in the dorsomedial (×0.3, $F_{(2,18)} = 27.226$, $p < 0.0001$ and ×0.4, $F_{(2,18)} = 27.226$, $p < 0.0001$, respectively), paraventricular (×0.6, $F_{(2,18)} = 43.780$, $p < 0.0001$ and ×0.6, $F_{(2,18)} = 43.780$, $p < 0.0001$, respectively) and ventromedial (×0.3, $F_{(2,18)} = 22.149$, $p < 0.0001$ and ×0.4, $F_{(2,18)} = 22.149$, $p < 0.0001$, respectively) nuclei compared to the vehicle. No difference was observed between effects of α-CZP and diazepam.

3.3.5. Amygdala

Globally in the amygdala α-CZP increased the number of c-Fos positive neurons compared to both the vehicle (×2.8, $F_{(2,18)} = 7.463$, $p = 0.0006$) and the diazepam (×1.8, $F_{(2,18)} = 7.463$, $p = 0.0206$). An increasing number of c-Fos positive neurons by α-CZP was particularly observed in the amygdalohippocampal, the basolateral, the medial and the posteromedial cortical nuclei. A difference was noted between the effects of α-CZP and diazepam but not between those of the peptide and the vehicle in the anterior cortical nucleus. Contrariwise, no effect of α-CZP or diazepam was observed in the anterior cortical, basomedial, central and posterolateral cortical nuclei.

3.3.6. Other regions

α-CZP decreased the number of c-Fos positive neurons in the bed nucleus of the stria terminalis (BNST; ×0.6, $F_{(2,18)} = 6.1655$, $p = 0.0015$) compared to the vehicle. Diazepam decreased the number of c-Fos positive neurons in the septum (×0.3, $F_{(2,18)} = 7.2480$, $p = 0.0005$) and in the caudate putamen (×0.3, $F_{(2,18)} = 3.7003$, $p = 0.0182$) compared to α-CZP.

4. Discussion

The present results show that the conflict generated by the LDB compared to a non-stressful situation in Swiss mice significantly increased c-Fos expression in the prefrontal cortices, hippocampal formation, accumbens nucleus, hypothalamus, septum, and BNST. These results are consistent with previous studies using an elevated plus-maze test (Duncan, Knapp, & Breese, 1996; Kovács, 1998), which is partly based on anxiogenic cues close to LDB (i.e. bright light and inescapable area). The i.p. injection of a reference anxiolytic molecule, diazepam at a dose of 1 mg/kg, modulated neural activity in the different regions of the CNS involved in anxiety. Our study indicates that a unique 1 mg/kg i.p. injection of α-CZP in Swiss mice also induced changes in neuronal activity of brain regions implicated in anxiety regulation. These changes happened only when the animal faced a situation generating anxiety, as the LDB-induced stressful situation in mice and were due to the anxiolytic-like properties of α-CZP that were confirmed in the present mouse model after an i.p. injection of 1 mg/kg. These anxiolytic-like properties resulted in the decrease in time spent in the dark area in the LDB test. Light area of LDB induces anxiety in Swiss mice, as injection of 1 mg/kg of diazepam reduces by 46% the time spent in the dark area of this apparatus (Bourin and Hascoët, 2003). Our results are in line with previous observation in rodents that identified an anxiolytic-like effect after i.p. injection of 0.4 mg/kg of α-CZP in the conditioned defensive burying test in rats (Miclo et al., 2001), an i.p. injection of 1 mg/kg in the elevated plus-maze in rats (Cakir-Kiefer et al., 2011), or after oral administration of 4.4 mg/kg in the elevated plus-maze in mice (Mizushige, Sawashi, Yamada, Kanamoto, & Ohinata, 2013).

The dose of 1 mg/kg diazepam used as reference anxiolytic molecule induced a reduction of c-Fos expression in most regions implicated in anxiety regulation both in the control (CS) and in the stressful situation (AIS). These results are in agreement with previous observation (Lkhagvasuren et al., 2014) that a single i.p. injection of 1 mg/kg diazepam in a control situation decreases c-Fos expression in only two location, the shell of the accumbens nucleus and the paraventricular nucleus of the hypothalamus, the latter being responsible for the glucocorticoid release regulation in stress-related situations (Herman & Cullinan, 1997). In contrast, in the stressful situation, a reduction of c-Fos expression in all the areas of the prefrontal cortex, the hippocampal formation, and the accumbens nucleus, as well as in the dorsomedial, paraventricular and ventromedial hypothalamic nuclei was observed after i.p. injection of 1 mg/kg diazepam, as described before (Beck & Fibiger, 1995; de Medeiros, Carlos Reis, & Eugênio Mello, 2005; Lkhagvasuren et al., 2014). Differences between studies are explained by the dose and the type of stimuli as different kinds of stimuli induce different c-Fos brain pattern sensitivity to anxiolytics (Beck & Fibiger, 1995; Kovács, 1998). The specificity of the effect of diazepam in the reduction of c-Fos expression has been explained by the density of $GABA_A$/BZD receptors in the brain (de Medeiros et al., 2005). Indeed, these receptors are highly localised in the prefrontal cortices, the hippocampal formation, the hypothalamus and the accumbens nucleus (Richards & Möhler, 1984). In the present study, a reduction of the c-Fos expression was observed after injection of 1 mg/kg diazepam in all these regions. However, despite a high abundance of these receptors also in the amygdala, no modulation of c-Fos expression in this area after diazepam injection was observed. This result was also witnessed in previous studies (de Medeiros et al., 2005; Lkhagvasuren et al., 2014; Panhelainen & Korpi, 2012; Salminen, Lahtinen, & Ahtee, 1996). This makes it possible to conclude that the conditions used were suitable for demonstrating the modulation of the neuronal activity by an anxiolytic molecule.

The present findings also provide neurobiological evidence supporting previous behavioural observations only done with CH in rats (Guesdon et al., 2006), dogs (Palestrini et al., 2010) or humans (Kim et al., 2007), suspecting that an anxious or stressful situation was needed to α-CZP for displaying anxiolytic-like properties. In the present study, in a non-stressful situation, i.e. the CS, a single i.p. injection of 1 mg/kg α-CZP did not affect c-Fos expression in any brain area. In contrast, in the AIS induced by the LDB, i.p. injection of 1 mg/kg α-CZP compared to the vehicle modified c-Fos expression in several brain regions. This included a reduction in the frontal association cortex, hippocampal formation especially CA2 and dentate gyrus, accumbens nucleus, dorsomedial, paraventricular and ventromedial nuclei of the hypothalamus as well as BNST. The global expression of c-Fos in this latter region will need to be refined as BNST is involved in a wide range of different functions. Moreover, an increase of about 185% in amygdala, and no global effect in the prefrontal cortices despite a local effect in the frontal association cortex were detected.

The results showed analogies and some differences between the action of 1 mg/kg α-CZP and diazepam in the central nervous system and, although the same dose was applied on a weight basis (1 mg/kg) for the two molecules, a dose difference effect when expressed on a molar basis (0.8 μmol/kg and 3.5 μmol/kg for α-CZP and diazepam, respectively) cannot also be excluded. Nevertheless, in behavioural tests, the reduction of the anxiety observed

after injection of 1 mg/kg of the peptide is absolutely the same that observed after administration of 1 mg/kg of diazepam despite a molar quantity ratio of 4 (Cakir-Kiefer et al., 2011). No effect of diazepam on c-Fos expression was observed in the amygdala whereas α-CZP increased this expression by about 185% in this area. Interestingly, the anxiolytic-like effect of 1 mg/kg α-CZP was associated with the absence of side effects sedation and addiction (Dela Peña et al., 2016; Messaoudi et al., 2009) that may be due to a selective binding of α-CZP on subtype(s) of $GABA_A$ receptors as α-CZP also displays an affinity for the BZD binding site of $GABA_A$ receptors (Miclo et al., 2001). Indeed, the anxiolytic properties of these receptors rely on the α_2 subunit (Löw et al., 2000) whereas the α_1 subunit carries the sedation action of the diazepam (Rudolph et al., 1999). $GABA_A$ receptor subtypes also show a specific distribution in the brain. $GABA_A$ α_2-containing receptors are mostly located in the hippocampal formation, as well as the amygdala, where α-CZP act on c-Fos expression. $GABA_A$ α_1-containing receptors are located in the prefrontal cortices (Fritschy & Mohler, 1995; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000) where α-CZP had no global impact on c-Fos expression since its effect was only detected in the frontal association cortex. An interaction of α-CZP with $GABA_A$ receptor subtypes other than those having the α_2 subunit cannot be ruled out because no data on the interaction of this peptide with $GABA_A$ receptor subtypes is available. Indeed, the affinity of the peptide for the benzodiazepine site of the $GABA_A$ receptor was determined in competition with flunitrazepam (Miclo et al., 2001). Flunitrazepam is a non-selective benzodiazepine that can bind to $GABA_A$ receptors that have either an α_1, α_2, α_3, or α_5 subunit, and which also demonstrated an affinity for receptors possessing the α_4 subunit, though $GABA_A$ receptors having an α_4 or α_6 subunit are said to be insensitive to diazepam (You, Kozuska, Paulsen, & Dunn, 2010).

In conclusion, the anxiolytic-like properties of α-CZP administrated by i.p. way at 1 mg/kg were confirmed in a Swiss mice model of anxiety using the LDB. Administration of this dose of α-CZP had somewhat similar effects as diazepam on neuronal activity modulation compared to vehicle in an anxiety-inducing situation and did not have any effect on neuronal activity compared to vehicle in a control situation (housing room). To our knowledge, a limited number of studies have demonstrated the modulation of neuronal activity via a peptide of food origin (Sun, Cade, Fregly, & Malcolm Privette, 1999) and this study can help to better understand the neurological effects of molecules produced during the digestion of food proteins. It can also be noted that our study has shown that the decapeptide, which was unmodified by intestinal digestion or absorption steps, since it has been administered intraperitoneally, is able to trigger this cerebral modulation. Nonetheless, whether it is the whole sequence or a shorter sequence which is responsible for this modulation remains to be determined. Indeed, the peptides YLGYLEQ (Cakir-Kiefer et al., 2011), YLG (Mizushige et al., 2013) or YL (Kanegawa, Suzuki, & Ohinata, 2010) have also previously shown an anxiolytic activity.

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Disclosures

During the course of the work, J. Schwarz was hired by Ingredia SA and is still employed by it. Ingredia SA partially financially supported the work. The funders had no role in the design and execution of the work. The remaining authors declare no competing financial interest.

Author contributions

SB, CC, CCK, DT, and LM conceived and designed the experiments. SB and CC performed the experiments. SB, CC, JS, CCK, DT, and LM analysed the data and wrote the paper. All authors have approved the final article.

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4 EVALUATION OF THE ANXIOLYTIC ACTIVITY OF, AND THE MODULATION OF BRAIN ACTIVITY BY YLGYL, A DERIVATIVE FROM α-CASOZEPINE

The objective of this second study was to evaluate the potential anxiolytic-like activity of YLGYL, a peptide derived from the *in vitro* digestion of α-CZP, in a murine model. YLGYL has been found in significant quantity in the hydrolysis medium of α-CZP by Corolase PP® or a combination of pepsin and Corolase PP®. The impact of this new peptide on the modulation of brain activity was also assessed in comparison with α-CZP and diazepam.

In view of the results obtained in the previous study, mice were this time only put in the anxiety-inducing situation triggered by the light/dark box 30 min after an i.p. injection of either vehicle, α-CZP, YLGYL, or diazepam. Mice thereafter underwent the same protocol as previously: mice were anesthetised and perfused *in situ* with formol, brains were harvested, frozen, and then sliced with a cryostat.

However, this time, coronal sections were chosen over sagittal ones, as some regions showing differences in the modes of action of α-CZP and diazepam were already identified in the previous study (i.e. prefrontal cortex and amygdala). Moreover, the use of coronal sections allowed an easier study of new regions situated in the brainstem which were also linked to anxiety regulation: the periaqueductal grey, the raphe nuclei, and the nucleus of the tractus solitarius. Neuronal activity was once again evaluated *via* the expression of the c-Fos protein revealed with immunofluorescence.

In summation

- The anxiolytic-like properties of YLGYL were also revealed after an i.p. injection in the murine model of the light-dark box.
- This peptide also modulates neuronal activity in brain regions linked to anxiety regulation while the results previously obtained with α-CZP were confirmed in this study with a new batch of mices.
- α-CZP and its derived peptide YLGYL do not show the exact same regulation of neuronal regulation:
    - α-CZP had increased neuronal activity globally in the amygdala while YLGYL is only efficient in some specific nuclei.
    - Neuronal activity was more increased in one of the raphe nuclei after the injection of YLGYL than after an injection of α-CZP.

Research article

YLGYL, a novel peptide derived from proteolysis of α-casozepine, displays anxiolytic-like properties with a different pattern of brain modulation than its precursor in mice.

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Short Title: YLGYL, a novel anxiolytic-like peptide.

Abbreviations: α-CZP, α-casozepine; **AIS**, anxiety-inducing situation; **BNST**, bed nucleus of the stria terminalis; **BZD,** benzodiazepine; **CH**, tryptic hydrolysate of bovine α_{s1}-casein; **GABA**, γ-amino butyric acid; **i.p.**, intraperitoneal; **LDB**, light/dark box; **NTS**, nucleus of the tractus solitarius; **PAG**, periaqueductal grey

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Tables and Figures: Tables: 1, Figures: 4

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ABTRACT

α-Casozepine (α-CZP) is an anxiolytic-like bioactive decapeptide derived from bovine α_{s1}-casein. The N-terminal peptide YLGYL, was previously identified after proteolysis of the original peptide in an *in vitro* digestion model. Its putative anxiolytic-like properties were evaluated in a Swiss mice model using a light/dark box (LDB) after an intraperitoneal injection (0.5 mg/kg). The effect of YLGYL on c-Fos expression in brain regions linked to anxiety regulation were afterwards evaluated via immunofluorescence and compared to those of α-CZP and diazepam, a reference anxiolytic benzodiazepine. YLGYL elicited some anxiolytic-like properties in the LDB, such as α-CZP and diazepam. The two peptides displayed some strong differences with diazepam in terms of c-Fos expression modulation in the prefontal cortex, the amygdala, the nucleus of the tractus solitarius, the periaqueductal grey, and the raphe magnus nucleus, implying a potential different mode of action. Additionally, YLGYL modulated c-Fos expression in the amygdala and in one of the raphe nuclei, displaying a somewhat similar pattern of activation as α-CZP. Nevertheless some differences were also spotted between the two peptides, making it possible to formulate the hypothesis that these peptides could have a different mode of action. Taken together, these results showed that YLGYL could participate to the *in vivo* overall action of α-CZP.

1. INTRODUCTION

A tryptic hydrolysate of bovine α_{s1}-casein exhibits anxiolytic-like properties after i.p. injection in rats (Miclo *et al*, 2001). The associated industrial formula displayed the same anxiolytic-like effects in rats after *per os* administration (Violle *et al*, 2006) as well as other animal species: cats, dogs, ponies and horses (Beata *et al*, 2007a, 2007b, McDonnell *et al*, 2013, 2014; Palestrini *et al*, 2010). Clinical trials revealed that this hydrolysate also had a positive influence on both physical and psychological anxiety symptoms in either healthy or stressed subjects (Kim *et al*, 2007; Lanoir *et al*, 2002; Messaoudi *et al*, 2005). Besides its anxiolytic-like properties, the hydrolysate demonstrated anticonvulsant effect in rats (Miclo *et al*, 2001) as well as sleep-protecting effects in rodents (Guesdon *et al*, 2006; Dela Peña *et al*, 2016). These properties brought the hydrolysate close to the benzodiazepines (BZDs) family, the most common anxiolytic drugs family, despite it did not show the side effects ordinarily associated to the BZDs such as habituation or sedation (Messaoudi *et al*, 2009; Dela Peña *et al*, 2016).

The screening of the different peptides of the tryptic hydrolysate on BZD site of $GABA_A$ receptors led to the discovery of α-casozepine (α-CZP), a decapeptide corresponding to the fragment 91-100 of bovine α_{s1}-casein (YLGYLEQLLR) (Miclo *et al*, 2001). This bioactive peptide also displayed anxiolytic-like effects in rats (Cakir-Kiefer *et al*, 2011b; Miclo *et al*, 2001) as well as in mice (Mizushige *et al*, 2013b). Recent findings have revealed that α-CZP can modulate neuronal activity, evaluated *via* c-Fos immunohistochemistry, in several brain regions linked to anxiety regulation in a mouse model, allowing a better understanding of its anxiolytic-like properties (Benoit *et al*, 2017).

An *in vitro* digestion model of α-CZP revealed that the peptide displays a certain resistance toward gastric and pancreatic proteases (pepsin, chymotrypsin, Corolase PP™, pepsin and

Corolase PP™) but the amount of peptide crossing the brush border may be not high enough to explain its activity. However a peptide was found in substantial proportion in the media after the action of these different proteases: YLGYLEQ (f91–97 of bovine α_{s1}-casein), which also displayed anxiolytic-like effects in rats in three behavioural tests (Cakir-Kiefer *et al*, 2011b). The absorption of this peptide through a Caco2 monolayer was facilitated in the presence of bile salts (Cakir-Kiefer *et al*, 2011a). The observed anxiolytic action may be due to a combined action of the two peptides, α-CZP and α_{s1}-CN-(f91-97).

In the study of Cakir-Kiefer et al., it was observed that the four peptide bonds belonging to the N-terminal part of α-CZP were totally resistant to the different proteases used (Cakir-Kiefer *et al*, 2011b). The resulting peptide, YLGYL, corresponding to fragment 91–95 of bovine α_{s1}-casein was also found in the hydrolysis media but in a lower amount than that of α_{s1}-CN-(f91-97). Since the bonds of this peptide are particularly resistant to proteolysis, it cannot be excluded that α_{s1}-CN-(f91-95) could also participate to the *in vivo* overall anxiolytic-like effects of α-CZP. The present study was then designed to evaluate YLGYL *in vivo* anxiolytic-like properties and its action on brain activity, by evaluating the expression of c-Fos, a neuronal activity marker (Chung, 2015; Kovács, 1998). Focus was set on brain areas involved in anxiety regulation, and more specifically on brain regions where differences were already spotted between α-CZP and diazepam, *i.e.* prefrontal cortex and amygdala (Benoit *et al*, 2017).

2. MATERIALS AND METHODS

2.1 Drugs

α-CZP (1 mg/kg i.e. 0.8 µmol/kg, Genosphere Biotechnologies, France), YLGYL (0.5 mg/kg i.e. 0.8 µmol/kg, Genosphere Biotechnologies, France) and diazepam (1 mg/kg i.e. 3.5 µmol/kg, Valium, Roche, Switzerland) were diluted in a 1% (v/v) glycerol, 0.2% (w/v) methylcellulose

aqueous solution. Each compound was injected by intraperitoneal (i.p.) way, in a volume of 5 mL/kg body weight. The control group received the same volume of vehicle.

2.2 Animals

All experiences were approved by the French "Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt" on the recommendation of the "Comité d'Éthique en Expérimentation Animale" of Jouy-en-Josas (N°02237.01). Male Swiss mice (n = 48) (Janvier Labs, France), aged of 9 weeks at arrival, were housed individually and maintained on a 12 h inversed light/dark cycle (lights off at 08:00 a.m.) and controlled environment (temperature 22±1°C, humidity 60%). Water and food were available *ad libitum*. Mice were handled every day by the same experimenter and particular care was taken to limit any kinds of external stress (variations in light, noise or odour). Behavioural tests were performed during their active period.

2.3 Light/dark box (LDB)

A homemade apparatus was used for the light/dark box procedure. Apparatus, experimental conditions and procedure, and collected data were defined according to Benoit et al. (Benoit *et al*, 2017).

To firstly assess the anxiolytic-like properties of YLGYL in the LDB model, animals (n = 8/group) were placed in the LDB, thirty minutes after an i.p. injection (5 mL/kg body weight) of either vehicle, α-CZP (1 mg/kg), YLGYL (0.5 mg/kg) or diazepam (1 mg/kg). Behaviours were recorded for five minutes and analysed before the beginning of the experimental procedure described below.

2.4 Neuronal activity evaluation procedure

Methods for neuronal activation evaluation using c-Fos immunofluorescence followed our previously described procedure (Benoit *et al*, 2017).

Briefly, after a week of acclimation to the lab conditions, mice received a soy protein-based diet (standard AIN-93M diet containing 20% of total energy as soy protein, 10% as fat and 70% as carbohydrate) in order to exclude the formation of endogenous casein-derived bioactive peptides. Two weeks later, animals received an i.p. injection (5 mL/kg body weight) of either vehicle, α-CZP (1 mg/kg), YLGYL (0.5 mg/kg) or diazepam (1 mg/kg) (n = 4/group). Thirty minutes later animals were placed in the LDB device for 5 minutes and thereafter back to their housing room.

Ninety minutes later, animals received a lethal i.p. injection of sodium pentobarbital (100 mg/kg, Ceva Santé Animale, France) and were subsequently perfused with 50 mL of DPBS supplemented with 0.05% (w/v) $NaNO_2$, followed by 100 mL of 4% (v/v) formaldehyde (Microm Microtech, France). Brains were harvested, cryo-protected in 15% (w/v) sucrose (Alfa Aesar, Germany) solution for 24 hours at 4°C and subsequently in 30% (w/v) sucrose solution at 4°C during 48 hours. They were afterwards snap frozen at –80°C and stored at –20°C until sectioning. Coronal sections (20 µm) were cut using a cryostat (CM1520, Leica, Germany) and stored at –20°C. They were rehydrated three times in PBS solution during 10 min. They were incubated in a PBS solution containing 0.5% (v/v) Triton X-100 and 2% (w/v) BSA during an hour at room temperature and subsequently incubated in a PBS / Triton X-100 / BSA solution containing in addition 1.5% (v/v) goat serum and rabbit anti-c-Fos antibody (primary antibody, 1:5000, Ab-5, Calbiochem, France) during 48 hours at 4°C. Slices were afterwards rinsed three times in PBS during 10 min and then incubated in a PBS / Triton X-100 / BSA solution with

Alexa-Fluor 488 (secondary antibody, 1:200, Molecular Probes, France) for 2 hours at room temperature. Eventually, slices were washed three times in PBS and mounted using a medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, France). Two c-Fos negative controls, obtained by omitting either the primary or the secondary antibody, were processed during each experiment to check for negative controls. No staining was observed on these two controls (data not shown). Slices were digitised using either a Lamina (amygdala only, Cochin HistIM Facility; Perkin Elmer, USA) using a ×20 objective lens and epifluorescence or an epifluorescence microscope (Axio Imager.Z1, Zeiss, Germany).

The same automated counting was used with ImageJ (Rasband, 1997) to evaluate the number of c-Fos neurons/0.04 mm² (Benoit *et al*, 2017). Brain regions were identified using a stereotaxic atlas (Paxinos and Franklin, 2001).

2.5 Statistical analysis

Behavioural and immunohistochemical results were analysed using one-way analysis of variance (ANOVA), after assessing the normal distribution of the residuals. Multiple comparison analysis was performed with Bonferroni post-hoc tests using R (R Core Team, 2013). All data are reported as mean ± SEM. Differences were considered to be significant at the $p < 0.05$ level.

3. RESULTS

Anxiolytic-like properties of YLGYL in the LDB. An i.p. injection of YLGYL at 0.5 mg/kg 30 min before the test increased the transitions between the two compartments (×2.1, $F_{(3,24)} = 4.8636$, $p = 0.0177$), the time spent in the lit box (×2.4, $F_{(3,24)} = 7.0194$, $p = 0.0034$) as well as the number of rears in the lit box (× 3.2, $F_{(3,24)} = 8.405$, $p = 0.0014$) compared to a

vehicle injection in the same conditions. α-CZP, injected at the same molar concentration, increased the time (×2.3, $F_{(3,24)} = 7.0194$, $p = 0.0043$) and the number of rears (×2.6, $F_{(3,24)} = 8.405$, $p = 0.0014$) in the lit box, while diazepam only increase the time spent in the lit box (×2.1, $F_{(3,24)} = 7.0194$, $p = 0.0243$) compared to the vehicle. LDB results are summed up in Figure 1.

Modulation of neuronal activity induced by intraperitoneal injection of either YLGYL, α-CZP or diazepam in an anxiety-inducing situation

YLGYL increased c-Fos expression in the anterior and posterior cortical nuclei of the amygdala (×2.8, $F_{(3,11)} = 21.771$, $p = 0.0012$ and ×2.4, $F_{(3,11)} = 53.659$, $p < 0.0001$, respectively) as well as the raphe magnus nucleus (RMg) (×3.3, $F_{(3,11)} = 82.797$, $p < 0.0001$) compared to the vehicle.

α-CZP increased c-Fos expression globally in the amygdala (×2.4, $F_{(3,11)} = 51.173$, $p = 0.0002$), and more specifically in the basolateral (×2.8, $F_{(3,11)} = 89.916$, $p < 0.0001$), the basomedial (×2.3, $F_{(3,11)} = 28.829$, $p = 0.0013$), and the medial (×2.9, $F_{(3,11)} = 61.17$, $p < 0.0001$) nuclei, compared to the vehicle. This peptide also increased c-Fos expression in the raphe magnus nucleus compared to the vehicle (×2.0, $F_{(3,11)} = 82.797$, $p = 0.0006$).

Eventually, only diazepam increased c-Fos expression in the nucleus tractus solitarius (NTS) (×2.4, $F_{(3,11)} = 8.5315$, $p = 0.0069$) and decreased it in the prefrontal cortex and periaqueductal grey (PAG) (×0.3, $F_{(3,11)} = 33.262$, $p < 0.0001$ and ×0.5, $F_{(3,11)} = 6.0371$, $p = 0.0309$, respectively) compared to the vehicle. Diazepam also increased c-Fos expression specifically in the central nucleus of the amygdala (×2.8, $F_{(3,11)} = 11.663$, $p < 0.0031$).

Some differences were also observed between YLGYL and α-CZP, as YLGYL displayed a higher c-Fos expression in the raphe magnus nucleus compared to α-CZP (×1.7, $F_{(3,11)} = 82.797$, $p = 0.0001$). c-Fos expression results are summed up in Table 1.

4. DISCUSSION

This study revealed that a unique 0.5 mg/kg (0.8 µmol/kg) i.p. injection of YLGYL in male Swiss mice triggered some anxiolytic-like properties in a LDB paradigm. Indeed, the reduction of the time spent in the dark area of this apparatus, which is an indicator of the anxiolytic-like properties of a molecule (Bourin and Hascoët, 2003), is similar to those of both α-CZP, injected at the same molar concentration, and diazepam, a benzodiazepine whose anxiolytic effect is well established. The results obtained with vehicle, α-CZP, and diazepam in Swiss mice in this device have already been published before (Benoit *et al*, 2017). The anxiolytic-like properties of α-CZP in rodents have already been well documented before (Cakir-Kiefer *et al*, 2011b; Miclo *et al*, 2001; Mizushige *et al*, 2013b). Thus, the shorter peptide, YLGYL, displayed a similar anxiolytic-like activity as the longer peptide, α-CZP. This has already been observed with the YLGYLEQ peptide, also derived from α-CZP (Cakir-Kiefer *et al*, 2011b). This suggest that an N-terminal pattern positioning the two tyrosine residues in a good conformation seems to be the active structure of the peptides, as it has already been mentioned before (Cakir-Kiefer *et al*, 2011b; Lecouvey *et al*, 1997). The longer C-terminal part of α-CZP may allow the maintenance of a suitable active conformation of the pattern implying the tyrosine residues despite the constraints caused by the addition of residues, and still be tolerated to exert the anxiolytic-like properties of the peptide. Since the peptides YLGYLEQ and YLGYL are formed in a modelled digestion process (Cakir-Kiefer *et al*, 2011b), the overall anxiolytic activity of α-CZP may be due to the addition of the activities of the three peptides. Some peptide structures may facilitate the passage of physiological barriers. However, it must be ensured that the

cerebral structures modulated by the peptides are the same. The dipeptide YL and the tripeptide YLG, which are contained in the three larger peptides display anxiolytic properties but the mode of action of these short peptides does not imply the benzodiazepine site of $GABA_A$ receptor (Kanegawa *et al*, 2010; Mizushige *et al*, 2013b) whereas α-CZP displays an affinity for this site (Miclo *et al*, 2001).

The i.p. injection of 1 mg/kg α-CZP induced changes in c-Fos expression after a challenge in an anxiety-inducing situation (i.e. the LDB) in the studied regions compared to a vehicle injection. The anxiety-inducing situation using an LDB was chosen as it was a way to trigger the anxiolytic-like properties of α-CZP and observe changes in c-Fos expression in brain regions linked to anxiety regulation (Benoit *et al*, 2017). The rationale behind this experimental paradigm is that anxious or stressful situations seem to be mandatory to trigger the anxiolytic-like properties of the tryptic hydrolysate of bovine α_{s1}-casein containing α-CZP, as it has already been observed in rats (Guesdon *et al*, 2006), dogs (Palestrini *et al*, 2010), or humans (Kim *et al*, 2007). It was then observed that, in this anxiety-inducting situation, a unique injection of α-CZP decreased c-Fos expression in hippocampal formation, accumbens nucleus, and dorsomedial, paraventricular and ventromedial nuclei of the hypothalamus, as well as bed nucleus of the stria terminalis (BNST), while it increased c-Fos expression in the amygdala (Benoit *et al*, 2017). This increased c-Fos expression in the amygdala was statistically different of the action of diazepam in this region. The results obtained in this study confirmed with a new set of animals the increased in c-Fos expression in the amygdala (more specifically in the basolateral, basomedial, medial and posterolateral cortical nuclei) after an i.p. injection of 1 mg/kg α-CZP compared to a vehicle injection. The absence of effect of α-CZP in the prefrontal cortex compared to the vehicle was also confirmed. On the other hand, diazepam, which served here as a positive control, displayed no effect in the amygdala, while decreasing

c-Fos expression in the prefrontal cortex, compared to the vehicle. These results were also coherent with the previously obtained results, using sagittal sections instead of coronal ones (Benoit *et al*, 2017). These patterns have been explained by the specific distribution of $GABA_A$ receptors in the brain, both diazepam and α-CZP having an affinity for the benzodiazepine fixation site (Miclo *et al*, 2001).

New results obtained in the NTS, PAG, and RMg also displayed some differences between α-CZP and diazepam. Indeed, α-CZP increased c-Fos expression in the RMg, while diazepam increased c-Fos expression in the NTS, and decreased it in the PAG, compared to the vehicle group. These three regions are located in the brainstem and are also linked to anxiety regulation. NTS serves as the relay of vagal afferences to the brain, and diazepam-sensitive $GABA_A$ receptors have been characterised in this region (Barron *et al*, 1997; Suzuki *et al*, 2004). PAG is the structure associated with freezing and escape behaviours (Carrive, 1993). Eventually, RMg belongs to the raphe nuclei system which innervates the forebrain with serotoninergic projections (Millan, 2003). These newly observed differences are in line with the hypothesis of a different mode of action between α-CZP and diazepam, which may explain the absence of side effects of the tryptic hydrolysate containing α-CZP (Messaoudi *et al*, 2009; Dela Peña *et al*, 2016).

Besides, the i.p. injection of YLGYL also impacted c-Fos expression in some of the studied regions. Indeed, the administration of the pentapeptide increased the number of c-Fos positive neurons in the anterior cortical and posterolateral cortical nuclei of the amygdala as well as in the RMg, compared to the vehicle. It then appears that YLGYL affects a subset of the cortical nuclei and not the basolateral, basomedial and medial nuclei of amygdala which also responded to the administration of α-CZP, while the increase observed in the RMg is significantly higher

than that of α-CZP. These differences between α-CZP and its derivate, YLGYL, can be interpreted by some differences in their modes of action. Indeed, the shorter length of YLGYL could mean that this peptide would either reach some other brain regions or even target some new receptors in the brain, as being less sterically hindered than α-CZP. Moreover, as YLGYL shows some similarities with RYLGYL, which has some affinity for opioid receptors (Loukas *et al*, 1983), and as the ArXXAr (where Ar is an aromatic residue) pattern seems to be crucial for the affinity of opiate peptides (Chang *et al*, 1981), the hypothesis of an involvement of the opioid system in the mode of action of YLGYL should be investigated. On the other hand, the differences observed between α-CZP and YLGYL in the modulation of c-Fos expression in the amygdala and the RMg, could also be insignificant in terms of the way the two peptides act. Further work is needed to explore both hypotheses.

Eventually, YLG and YL peptides have been previously shown to also trigger anxiolytic-like properties in rodents (Kanegawa *et al*, 2010; Mizushige *et al*, 2013a, 2013b). YLG correspond to the fragment 91-93 of bovine α_{s1}-casein and is released after a pepsin-pancreatin *in vitro* digestion of α_{s1}-casein (Mizushige *et al*, 2013b). The anxiolytic-like properties of both YL and YLG was blocked by antagonists for serotoninergic 5-HT_{1A}, dopamine D_1, and $GABA_A$ receptors without a direct agonist action on these receptors (Kanegawa *et al*, 2010; Mizushige *et al*, 2013b). The anxiolytic-like properties of YL were also shown to not be reliant on opioid receptors (Kanegawa *et al*, 2010). On the other hand, α-CZP was selected for its affinity on the benzodiazepine site of $GABA_A$ receptors (Miclo *et al*, 2001), confirmed with a direct action on these receptors (Dela Peña *et al*, 2016). The loss of the YXXY pattern in a peptide could then mean a potential loss of the benzodiazepine site affinity, as the distance between the centres of the two tyrosine aromatic rings has been shown to be similar to that between the centres of the aromatic rings in nitrazepam, a benzodiazepine (Lecouvey *et al*, 1997). Moreover, the loss of

the this pattern could also mean a potential loss of affinity for opioid receptors (Chang *et al*, 1981). In summary, it could then be hypothesised that all the peptides derived from α-CZP may not have the same mode of action in terms of target receptors or neurotransmitters involved.

In conclusion, a unique i.p. injection of YLGYL, an α-CZP derivative, generated by Corolase PP™ or pepsine/Corolase PP™ hydrolysis, triggered anxiolytic-like properties in a Swiss mice model using the LDB paradigm at a molar concentration similar to α-CZP. The effect of α-CZP on neuronal activity in the prefrontal cortex and amygdala, while animals were put in an anxiety-inducing situation, was confirmed in this study, while some new differences were spotted between the peptide and a reference benzodiazepine, diazepam. The administration of YLGYL had also fairly similar effects as α-CZP on neuronal activity, compared to vehicle and diazepam in the same situation. Some differences in the amygdala and raphe nuclei were still pointed out between the two peptides and further pharmacological studies would help understand the mode of action of these food-derived anxiolytic-like peptides.

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DISCLOSURES

During the course of the work, J. Schwarz was hired by Ingredia SA and is still employed by it. Ingredia SA partially financially supported the work. The funders had no role in the design and execution of the work. The remaining authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

SB, CC, CCK, DT, and LM conceived and designed the experiments. SB and CC performed the experiments. SB, CC, JS, CCK, DT, and LM analysed the data and wrote the paper. All authors have approved the final article.

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Table 1. The effects of an i.p. injection of α-CZP (1 mg/kg), YLGYL (0.5 mg/kg) or diazepam (1 mg/kg) on the anxiety-induced c-Fos immunoreactivity (positive cells/0.04 mm²) in different areas of mice brains (n = 4/group). Data are mean ± SEM. Results were analysed using a one-way ANOVA to detect the effects of treatment (*p < 0.05, **p < 0.01, ***p < 0.001). Subsequently, a Bonferron post-hoc test was performed to compare the effect of α-CZP, YLGYL and diazepam to the vehicle (†p < 0.05, ††p < 0.01, †††p < 0.001).

	Vehicle	α-CZP	YLGYL	Diazepam	ANOVA	
					$F_{(3,11)}$	(p-value)
Prefrontal cortices						
Global	7.59 ± 0.61	6.47 ± 0.27	5.81 ± 0.41	2.15 ± 0.16 †††	33.262	8.21E-06 ***
Frontal association cortex	4.45 ± 0.33	4.92 ± 0.18	3.86 ± 0.49	1.85 ± 0.24 †††	14.661	3.69E-04 ***
Prelimbic cortex	8.28 ± 0.60	7.84 ± 0.85	6.98 ± 0.42	2.49 ± 0.14 †††	27.389	2.11E-05 ***
Medial orbital cortex	11.18 ± 1.09	9.15 ± 0.60	10.52 ± 0.96	2.91 ± 0.13 †††	22.654	5.19E-05 ***
Ventral orbital cortex	10.03 ± 0.90	8.12 ± 1.19	9.87 ± 0.74	2.96 ± 0.09 †††	19.242	0.00011 ***
Lateral orbital cortex	8.56 ± 0.85	6.54 ± 0.62	7.28 ± 0.50	1.83 ± 0.17 †††	25.734	2.84E-05 ***
Dorsolateral orbital cortex	6.05 ± 0.23	5.77 ± 0.29	5.32 ± 0.52	1.45 ± 0.05 †††	45.99	1.63E-06 ***
Amygdala						
Global	2.56 ± 0.14	6.21 ± 0.08 †††	3.64 ± 0.34	2.16 ± 0.39	51.173	9.47E-07 ***
Anterior cortical nucleus	3.65 ± 0.31	7.82 ± 1.30 †	10.25 ± 1.00 ††	1.54 ± 0.21	21.771	6.25E-05 ***
Posterolateral cortical nucleus	3.71 ± 0.49	7.39 ± 0.29 †††	8.93 ± 0.73 †††	1.72 ± 0.29 †	53.659	7.43E-07 ***
Basolateral nucleus	1.49 ± 0.05	4.17 ± 0.11 †††	1.91 ± 0.15	1.11 ± 0.23	89.916	5.07E-08 ***
Basomedial nucleus	2.88 ± 0.39	6.60 ± 0.38 †††	3.41 ± 0.54	1.78 ± 0.33	28.829	1.65E-05 ***
Central nucleus	2.33 ± 0.41	4.17 ± 0.20	1.93 ± 0.25	6.59 ± 1.25 ††	11.663	0.00133 **
Medial nucleus	3.29 ± 0.26	9.38 ± 0.49 ††	4.48 ± 0.62	2.63 ± 0.24	61.17	3.79E-07 ***
Nucleus of the Tractus Solitarius	9.03 ± 1.54	11.25 ± 1.90	8.42 0.64	21.24 ± 3.45 ††	8.5315	0.00328 **
Periaqueductal Grey	6.68 ± 0.65	6.01 ± 0.96	7.03 ± 0.86	3.13 ± 0.53 †	6.0371	0.01101 *
Raphe magnus nucleus	1.61 ± 0.17	3.18 ± 0.05 †††	5.35 ± 0.43 †††	1.20 ± 0.08	82.797	7.83E-08 ***

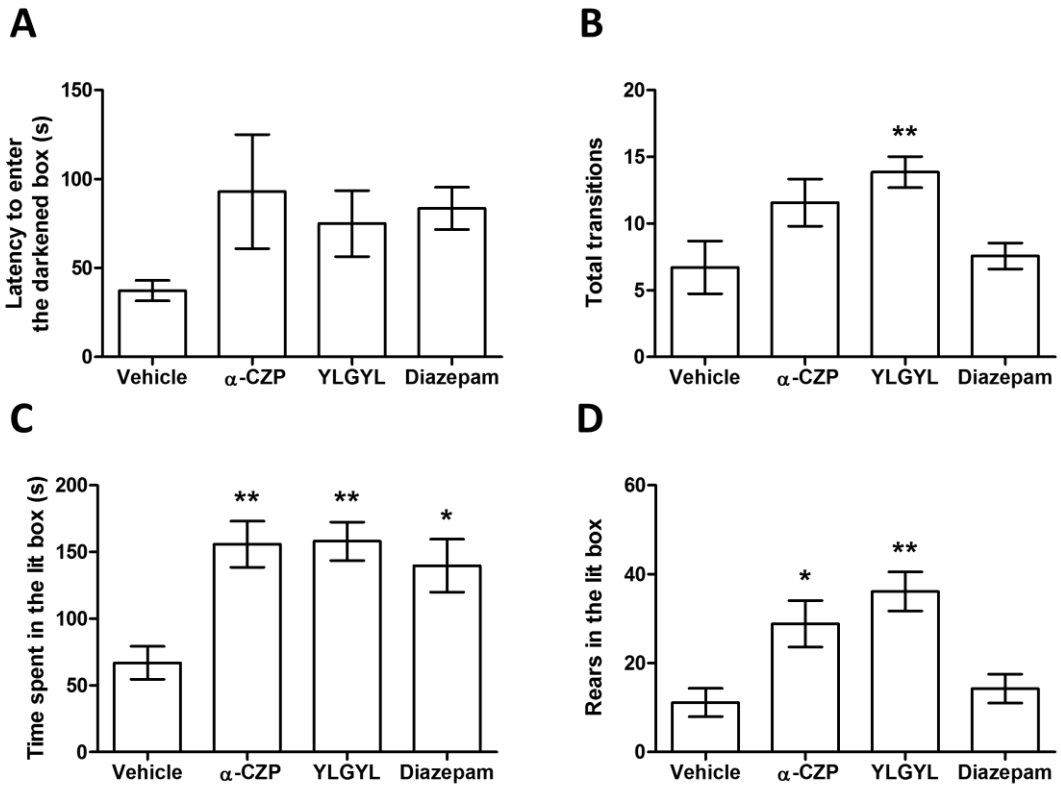

Figure 1. The effects of an i.p. injection of α-CZP (1 mg/kg), YLGYL (0.5 mg/kg) and diazepam (1 mg/kg) on the behavioural response in the light/dark box in Swiss mice. Results were analysed using a one-way ANOVA to detect the effect of the treatment on the studied behavioural scores. Data are mean ± SEM (n = 7-8/group). *p < 0.05, **p < 0.01 compared to the vehicle

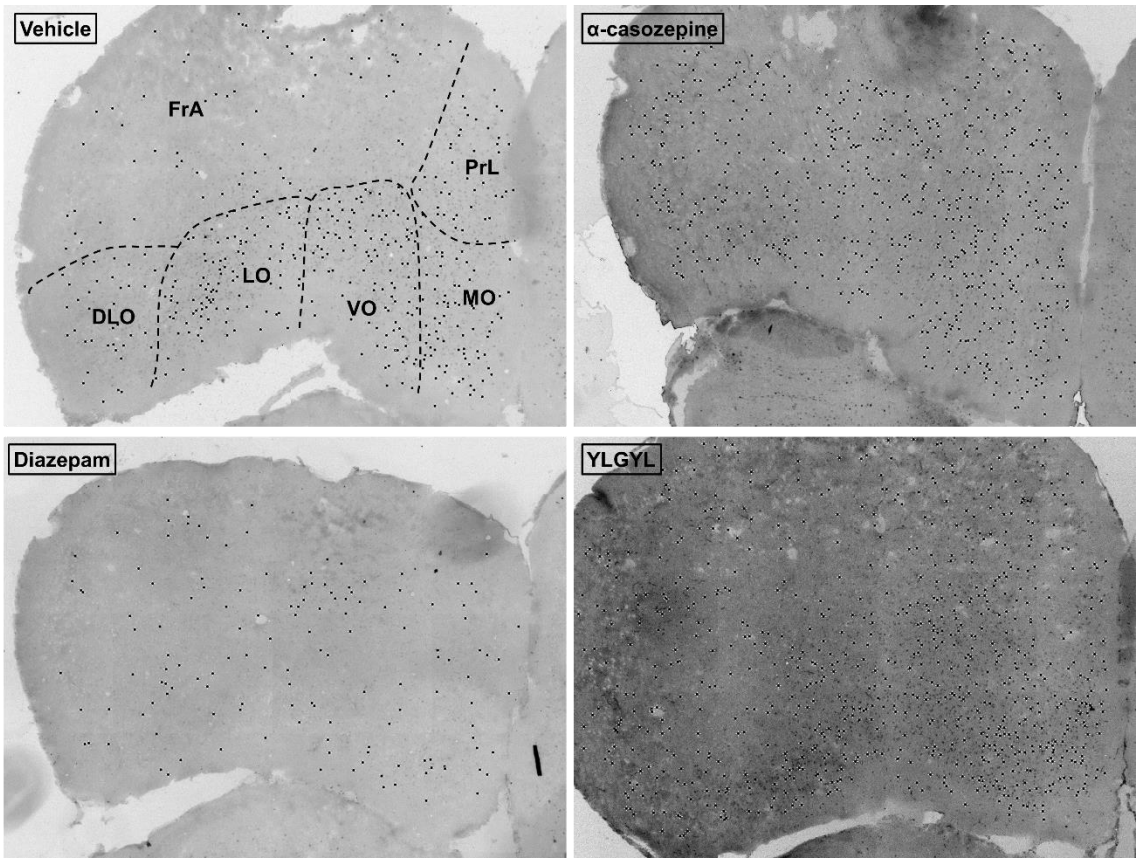

Figure 2. Photomicrographs illustrating the effect of the vehicle, α-CZP (1 mg/kg, i.p.), YLGYL (0.5 mg/kg, i.p.), and diazepam (1 mg/kg, i.p.) on anxiety-induced c-Fos immunoreactivity on the prefrontal cortex (Bregma –1.58 mm). Scale bar: 500 μm. FrA: frontal association cortex; PrL: prelimbic cortex; DLO: dorsolateral orbital cortex; LO: lateral orbital cortex; VO: ventral orbital cortex; MO: medial orbital cortex.

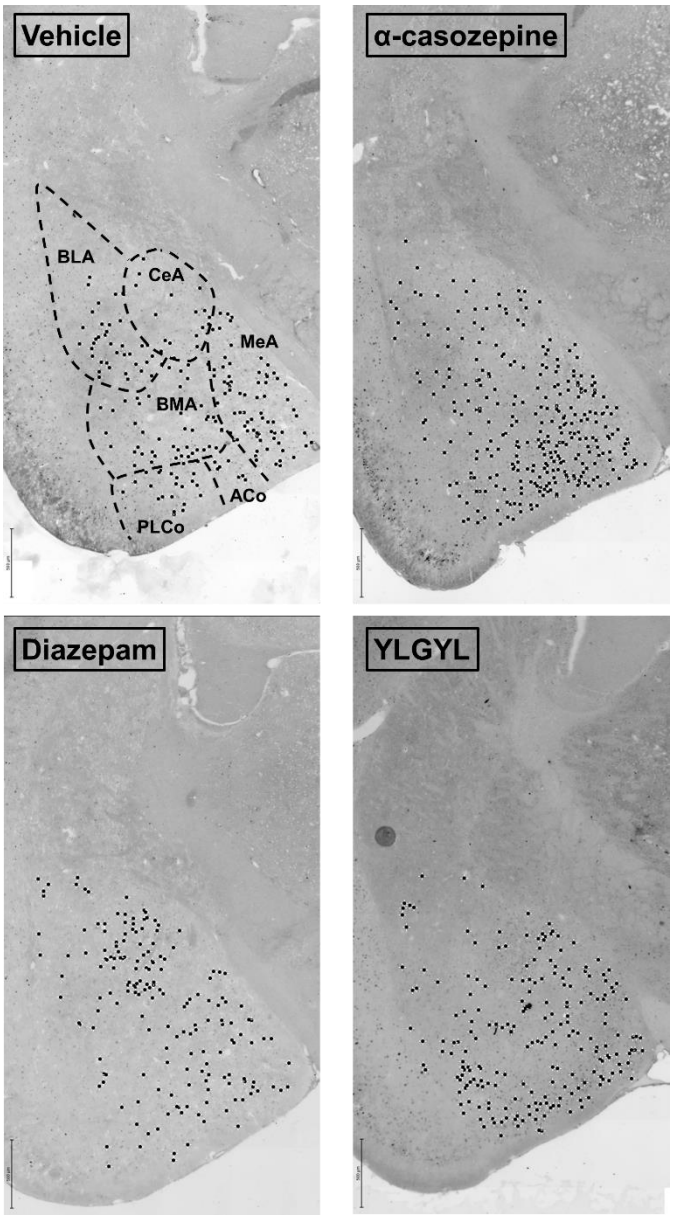

Figure 3. Photomicrographs illustrating the effect of the vehicle, α-CZP (1 mg/kg, i.p.), YLGYL (0.5 mg/kg, i.p.), and diazepam (1 mg/kg, i.p.) on anxiety-induced c-Fos immunoreactivity on the amygdala (Bregma 2.68 mm). Scale bar: 500 μm. BLA: basolateral nucleus; CeA: central nucleus; BMA: basomedial nucleus; PLCo: posterolateral cortical nucleus; ACo: anterior cortical nucleus; MeA: medial nucleus.

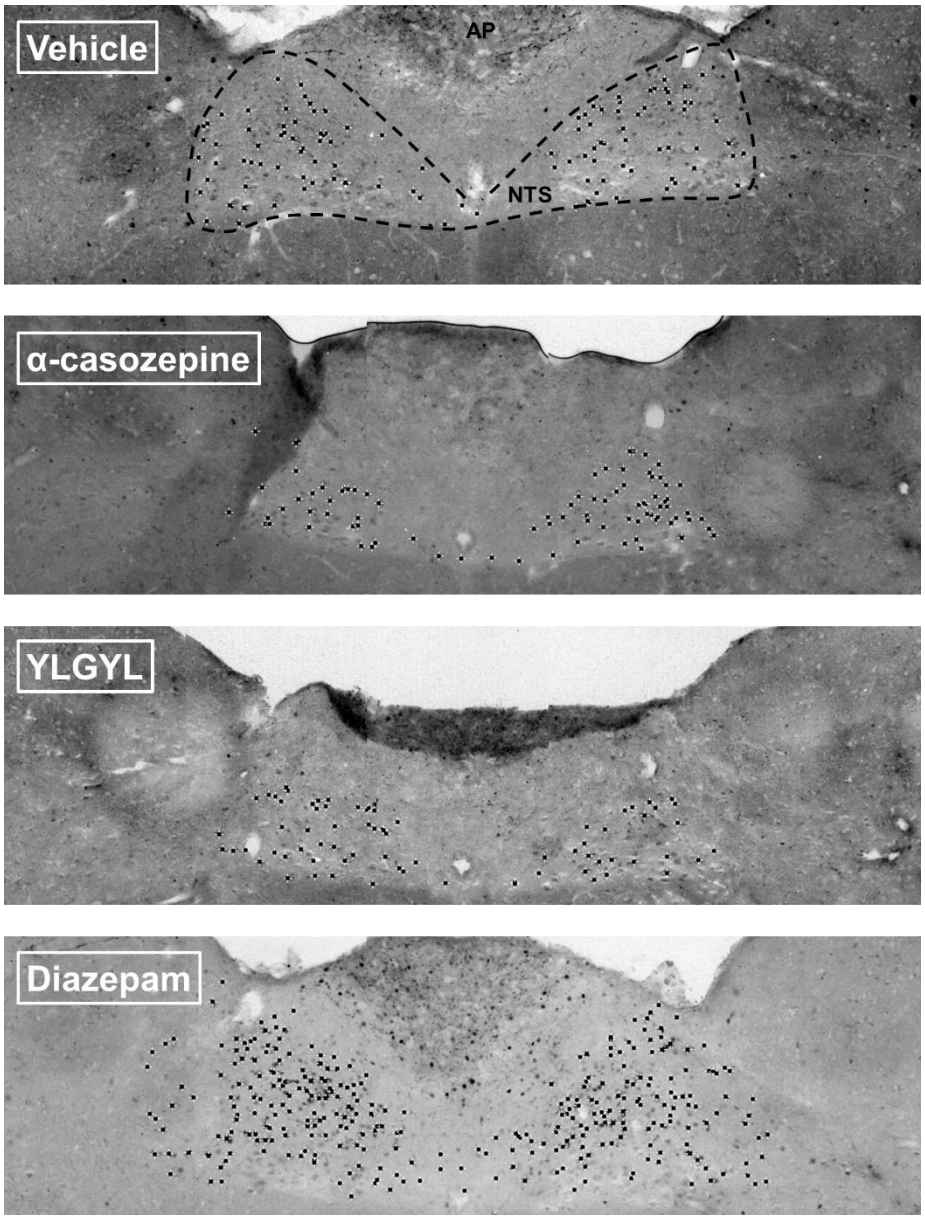

Figure 4. Photomicrographs illustrating the effect of the vehicle, α-CZP (1 mg/kg, i.p.), YLGYL (0.5 mg/kg, i.p.), and diazepam (1 mg/kg, i.p.) on anxiety-induced c-Fos immunoreactivity on the nucleus of the tractus solitarius (Bregma –1.58 mm). Scale bar: 500 μm. AP: area postrema; NTS: nucleus of the tractus solitarius.

5 INVESTIGATIONS CONCERNING THE MEDIATION OF ANXIOLYTIC-LIKE PROPERTIES OF α-CASOZEPINE

After understanding which brain regions were associated with the anxiolytic-like properties of α-CZP, a next step would be to understand how these properties are mediated. Two lines of investigation were then initiated: how are the anxiolytic-like properties of α-CZP mediated from the gastrointestinal tract to the brain and which neurotransmitters are involved in these same properties? Three sets of experiments were then designed to partly answer these questions.

Two major ways of communication exist between the gastrointestinal tract and the brain: the blood circulation and the vagus nerve. A first experiment was composed to understand the role of the latter in the mediation of the anxiolytic-like properties of the industrial tryptic hydrolysate of bovine α_{s1}-casein containing α-casozepine (iCH). Rats underwent a complete subdiaphragmatic vagotomy surgery and were then gavaged with iCH. Their behaviour was thereafter characterised in a conditioned defensive burying paradigm and compared with sham animals also administered with iCH as well as control animals which did not underwent the surgery nor received iCH.

Two other sets of experiments were designed to understand the role of two neurotransmitters' receptors, GABA type A receptor ($GABA_A$) and serotonin type 1A receptor ($5\text{-}HT_{1A}$), in the mode of action of both iCH and α-CZP. Antagonists of these receptors (bicuculline and NAN-190, respectively), were i.p. injected with either an oral administration of iCH or an i.p. injection of α-CZP. Behaviours were evaluated using a conditioned defensive burying model. Diazepam was used as a positive control for iCH and α-CZP and tested within the same experimental paradigm.

All these experiments are derived from 2000, 2006, and 2009 reports originating from the collaboration between Ingredia and ETAP-Lab.

The results obtained in the study have been submitted to *Molecular Nutrition and Food Research* on July 15, 2016:

Benoit, S., Chaumontet, C., Cakir-Kiefer, C., Tomé, D., Miclo, L., Violle, N., Schwarz, J. Evaluation of the involvement of vagus nerve, 5-HT_{1A} and $GABA_A$ receptors in the anxiolytic-like properties of a tryptic hydrolysate of bovine α_{s1}-casein containing α-casozepine.

The response of the Editor was received on September 2, 2016 and was a refusal. Despite some positive comments on the novelty of the experiments (especially the vagotomy part) and the perspective they bring to the understanding of the mode of action of iCH and α-CZP, as well as the way the manuscript was written and illustrated, some concerns were raised by the two reviewers.

One was concerning the ways of administration and the dose of iCH and α-CZP. Indeed iCH was orally administered at the dose of 15 mg/kg, while α-CZP was i.p. injected at the dose of 0.8 mg/kg. The second dose being then higher than the theoretical dose of α-CZP administered after oral gavage of iCH. Indeed, α-CZP represents 5,3% of bovine α_{s1}-casein (0,8 mg for 15 mg) but represents 1,8% of the peptide content of iCH (0,3 mg for 15 mg).

Another one was concerning the choice of the antagonists as bicuculline is a global $GABA_A$ antagonist while NAN-190 has been shown to also have an affinity for α_2-adrenergic receptors. Eventually, the last and biggest concern was that some negative controls were missing from the experimental paradigm. Indeed, the controls with only the injection of the antagonist and of the solution of interest (either iCH, α-CZP, or diazepam) were absent of the original reports. Without these information, it was then difficult to conclude of the results as bicuculline and NAN-190 may also have some anxiolytic-like of anxiogenic-like properties on their own.

Consequently, the pharmacological experiments were repeated with most of the comments taken into account. All the negative controls (i.e. injection of the antagonists with vehicle) were this time included in the experimental design. Flumazenil was used instead of bicuculline as this new antagonist is specific for the BZD site of the $GABA_A$ receptor. NAN-190 was however conserved as most of the available antagonists for 5-HT_{1A} also show some non-specificity (e.g. WAY-100,635 is a full dopamine D4 receptor agonist while WAY-100,135 is a partial 5-HT_{1B} and 5-HT_{1D} agonist). Only iCH was tested in this newer paradigm yet. Data are still under analysis.

Evaluation of the involvement of vagus nerve, 5-HT_{1A} and $GABA_A$ receptors in the anxiolytic-like properties of a tryptic hydrolysate of bovine α_{s1}-casein containing α-casozepine.

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Abbreviations: α-CZP: α-casozepine; Bic: bicuculline; CH: tryptic hydrolysate of α_{s1}-casein from bovine milk; GABA: γ-aminobutyric acid; $GABA_A$ receptor: γ-aminobutyric acid type A receptor; *i.p.*: intraperitoneal; $5\text{-}HT_{1A}$ receptor: 5-hydroxytryptamine (serotonin) 1A receptor

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Abstract

Scope: A tryptic hydrolysate of bovine α_{s1}-casein (CH) exerts anxiolytic-like properties in many animal species including humans. This was mainly related to the presence of α-casozepine (α-CZP), which yields these properties in rats. This study evaluated in a rat model, the role of the vagus nerve in the mode of action of CH and those of 5-HT_{1A} and $GABA_A$ receptors in the anxiolytic-like properties of both CH and α-CZP.

Methods and results: The conditioned defensive burying test was used to evaluate anxiety. Participation of the vagus nerve in the mode of action of CH was excluded, as the global score of anxiety in vagotomised rats was not significantly different than that of non-vagotomised animals. Both CH and α-CZP anxiolytic-like properties were eliminated by blocking 5-HT_{1A} receptors. Inhibition of $GABA_A$ receptors only had a tendency to suspend α-CZP anxiolytic-like action.

Conclusion: The physiological targets of CH and α-CZP could be localised at a different level than the gut. The slight difference, concerning $GABA_A$, observed between CH and α-CZP may be explained by administration ways. Intraperitoneal injection of α-CZP may preserve its structure and thereby its affinity for $GABA_A$. A change in peptide composition of the hydrolysate during digestion could also be involved.

1. Introduction

Hydrolysis of milk proteins releases different peptides, some of which could manifest bioactive properties (Hernández-Ledesma *et al*, 2013). A tryptic hydrolysate of α_{s1}-casein from bovine milk exerts anxiolytic-like and anti-convulsivant effects in rats (Miclo *et al*, 2001) and its industrial counterpart (CH, Lactium®) exhibits the same anxiolytic-like effects in rats (Violle *et al*, 2006), and in humans (Kim *et al*, 2007; Messaoudi *et al*, 2005), as well as sleep-modulating properties in rats (Guesdon *et al*, 2006). Anxiolytic-like properties of CH were confirmed amongst other animals such as cats (Beata *et al*, 2007a), dogs (Beata *et al*, 2007b), ponies (McDonnell *et al*, 2013) and horses (McDonnell *et al*, 2014). The anxiolytic-like properties were associated to the presence of the 91-100 decapeptide fragment of bovine α_{s1}-casein (YLGYLEQLLR) called α-casozepine (α-CZP) which displays these effects in rats (Miclo *et al*, 2001). *In vitro* digestion of α-CZP revealed that a shorten peptide derived from α-CZP (YLGYLEQ) also possess anxiolytic-like properties in the rat (Cakir-Kiefer *et al*, 2011b), and could then contribute to the *in vivo* property of α-CZP.

Anxiety is a complex phenomenon involving several brain regions (Steimer, 2002) and different neurotransmitter systems including the GABAergic system, the target of the benzodiazepines, the most prescribed anxiolytic drugs (Rudolph and Knoflach, 2011) and the serotoninergic system with 5-HT_{1A} receptors (Millan, 2003; Olivier *et al*, 2013) as new target for anxiolytic drugs (Parks *et al*, 1998). The decapeptide α-CZP exhibits affinity for the benzodiazepine site of $GABA_A$ receptors but this one is 10,000 times lower than that of the benzodiazepine diazepam (Dzp) (Miclo *et al*, 2001), while CH effect on chloride ion influx in neuroblastoma cell culture was blocked by bicuculline, a GABAA receptor antagonist (Dela Peña *et al*, 2016). Compared to Dzp, CH did not induce memory-impairment, tolerance or dependence side effects in rats (Messaoudi *et al*, 2009).

The aim of this study was to better understand the mechanism by which CH and α-CZP display anxiolytic-like activities. A number of arguments support that the mode of action of α-CZP could be central. Nevertheless, the question of a peripheral mechanism of action in the intestine remains. Indeed, as recently shown, the gut-brain axis and especially the vagus nerve plays a role in anxiety regulation. Vagal afferents modulates neurotransmitters in key areas of the limbic system (Klarer *et al*, 2014) and the anxiolytic-like effects of a probiotic orally administered are mediated by the vagus nerve (Bercik *et al*, 2011; Bravo *et al*, 2011). Thus, a first experiment assessed the activity of an orally administered single dose of CH after subdiaphragmatic vagotomy in rats. A second experiment assessed the implication of 5-$HT1_A$ and $GABA_A$ receptors in the anxiolytic-like activities of CH and α-CZP by using the receptors antagonists NAN-190 (Glennon *et al*, 1988) and bicuculline, respectively. The anxiolytic-like properties of CH and α-CZP are evaluated using the conditioned burying test, based on Pinel and Treit's work (Treit *et al*, 1981), as the anxiolytic-like activities of both hydrolysate and peptide were well assessed in this procedure (Miclo *et al*, 2001; Violle *et al*, 2006).

2. Materials and methods

2.1. Animals

All experiments adhered to the guidelines provided by the ASAB Ethical Committee for the use of animals in research (Animal Behavior 1993; 45:209-12) and by the Canadian Council on Animal Care and all procedures are in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Experiments were conducted before 2010 legislation. The assessment of the activity of orally administered CH after subdiaphragmatic vagotomy (Experiment 1) was carried out using 24 male Wistar rats (HsdBrlHan, Harlan, The Netherlands), weighing 250–275 g at their arrival. The experiment using receptor antagonists (Experiment 2) was carried out on 152 male Wistar AF EOPS (Centre Iffa-Credo, France),

weighing 300–320 g at their arrival. Rats were housed four per cage in polycarbonate cages 48 × 27 × 20 cm (U.A.R., France) in a regulate environment (humidity 55 ± 10%; temperature 22 ± 1°C; lights off: 08:00 a.m. – 08:00 p.m.). Rats were allowed free access to food (food pellets M20, Dietex, France for Experiment 1 or food pellets M25, Extralabo, France for Experiment 2) and tap water until the day before the experiments. After an acclimation period of 7 days after the day of their arrival, the rats were randomly assigned into three groups (n = 8/group) for Experiment 1 and fourteen groups for Experiment 2 (n = 8-12/group). The rats in the different groups were all handled in the same way and under the same conditions.

2.2. Experimental procedure

2.2.1. Experiment 1

Sixteen animals were anesthetised with an *i.p.* injection of 2 mg/kg acepromazine maleate (Calmivet, Vétoquinol, France) followed by an *i.p.* injection of 50 mg/kg ketamine (Virbac, France), and then subjected to a complete subdiaphragmatic vagotomy (n = 8) or sham operation (n = 8). Briefly, after laparotomy, the two trunks of the vagus nerve were identified under an operating microscope. Both trunks were cut off close to the diaphragm. For sham vagotomy, the vagus nerve was similarly exposed but was not cut. After surgery, a recovery period of 3 weeks was allowed before the conditioned defensive burying test (described below). Three days after the conditioned defensive burying test, success of the vagotomies was verified by the measure of food intake after an *i.p.* injection of the neuropeptide CCK-8S (Sigma, France), at a dose of 4 µg/kg. CH (Lactium®, Ingredia, France) was dissolved at 3 mg/mL in distilled water subsequently before an oral administration at a single dose of 15 mg/kg one hour before testing.

2.2.2. Experiment 2

CH (Lactium®), synthetic α-CZP (NeoMPS, France), Dzp (Roche, France), NAN-190 (5-HT_{1A} antagonist, Tocris, UK) and bicuculline ($GABA_A$ antagonist, Sigma, France) were suspended in 1% (w/v) methylcellulose solution with a magnetic stirrer for 30 minutes. Compounds were prepared just before their administration. Groups and used products are summed up in Table 1. To evaluate activity of CH after oral administration, NAN-190 or bicuculline (Bic) was *i.p.* injected 65 minutes before the test at a dose of 3 mg/kg, whereas CH or Dzp was orally administered 60 minutes before the test (15 mg/kg and 3 mg/kg, respectively). Concerning that of α-CZP after *i.p.* injection, NAN-190 or Bic was *i.p.* injected 45 minutes before the test at a dose of 1 mg/kg, whereas α-CZP or Dzp was *i.p.* injected 30 minutes before the test (0.8 mg/kg and 1 mg/kg, respectively). Concerning activity of Dzp after *i.p.* injection in presence of receptors antagonists, NAN-190 or Bic was administered *i.p.*, 45 minutes before the test at a dose of 1 mg/kg whereas Dzp was administered 30 minutes before the test (1 mg/kg, *i.p.*). Volumes administered were 2 mL/kg *i.p*. and 3 mL/kg orally.

2.3. Conditioned defensive burying Test

2.3.1. Apparatus

The conditioned defensive burying test used in the present study is based on Pinel and Treit's procedure (Treit *et al*, 1981). Habituation, shocking and testing were done in a 45 × 30 × 20 cm clear Plexiglas chamber, which floor was evenly covered by 5 cm of bedding material made of wood sawdust. On the centre of one wall, 2 cm above the level of the bedding material, a shock-probe could be inserted *via* a small hole. The shock probe consisted of a 7 × 2 × 0.5 cm Plexiglas slide overlaid with a copper wire-integrated circuit connected to an electric shock generator, which can deliver 0 to 8 mA (OPEN-Systems, France). The release was manually handled by

the operator. In the slightly lit test room, a CCD-TV camera allowed the rats to be observed and recorded from a neighbouring room.

2.3.2. *Procedure*

For habituation to the experimental conditions, each home cage group was placed in the test chamber without the shock-probe for 20 minutes during the two days prior to the test. The shock-probe was inserted into the chamber before the test session. Rats were individually placed in the test chamber, on the side opposite to the shock-probe. The first time the rat touched the probe with its forepaws, the experimenter delivered a single 2 mA shock. Immediately after shock administration, the behaviour of the rat was recorded for 5 minutes. Bedding material was changed each day of testing and smoothed to a uniform depth of 5 cm between each rat test. Rats that did not touch the probe within 5 minutes were excluded from the study. The conditioned defensive burying test was performed during the first 3 hours of the dark cycle, which is the period where the rats are more active.

2.3.3. *Studied parameters*

The following parameters were scored by an experimenter unaware of the group setup: 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. The "number of approaches" and the "number of retreats" allowed to calculate the "percentage of approaches towards the probe followed by retreats":

$$\frac{number\ of\ retreats}{number\ of\ approaches} \times 100$$

Within each of the variables "duration of probe-burying", "number of head stretchings towards the probe" and "percentage of approaches towards the probe followed by retreats", all values

were classified in increasing order and then transformed in their respective ranks. For each rat, the sum of the ranks of the three variables represents its global score of anxiety.

2.4. Statistical analysis

All data are expressed in percentage to the vehicle group (100%) and are reported as mean ± SEM. Data were evaluated with one-way analysis of variance (ANOVA). Multiple comparison analysis was performed with Tukey HSD post hoc tests using R (R Core Team, 2013). Differences were considered to be significant at $p < 0.05$.

3. Results

Anxiolytic properties of CH after oral administration in vagotomised rats

Compared to vehicle, CH orally administered at a single dose of 15 mg/kg significantly decreased the global score of anxiety of sham male Wistar rats (−45%, $p = 0.0161$; Figure 1) measured in conditioned defensive burying test. A significant decrease of the global score of anxiety compared to the vehicle group was also observed for the vagotomised rats (−52%, $p = 0.0444$). No difference between sham operated and vagotomised rats receiving CH was detected (+13%, $p = 0.9268$). Surgery was a success for all vagotomised animals as it was shown that food intake did not decrease after an *i.p.* injection of CCK-8S (data not shown), CCK satiating action being mediated by the vagus nerve (Moran *et al*, 1997).

Effects of 5-HT_{1A} or $GABA_A$ receptor antagonists on anxiolytic properties of CH after oral administration

Animals in both the Dzp and CH groups showed a significantly lower global score of anxiety in comparison to rats that received the vehicle (−75%, $p = 0.0034$ and −61%, $p = 0.0212$, respectively; Figure 2). CH/NAN-190 treated rats had a significantly higher global score of anxiety than rats in the CH group (+208%, $p = 0.0001$). No difference was observed compared

to the Control group. On the other hand, rats in the CH/Bic group showed no difference with rats in the CH or Control group.

Effects of 5-HT$_{1A}$ or GABA$_A$ receptor antagonists on anxiolytic properties of α-CZP after i.p. injection

Animals receiving Dzp or α-CZP displayed a significant lower global score of anxiety in comparison with Control rats (−89%, $p < 0.0001$ and −74%, $p = 0.0004$, respectively; Figure 3). Animals in the α-CZP/NAN-190 group showed a significant higher global score of anxiety in comparison with rats in the α-CZP group (+265%, $p = 0.0155$). No difference was observed with the Control group. On the other hand, α-CZP/Bic animals displayed a tendency to increase the global score of anxiety compared to the α-CZP group (+214%, $p = 0.0769$). No difference was observed between α-CZP/Bic and Control groups.

Effects of 5-HT$_{1A}$ or GABA$_A$ receptor antagonists on anxiolytic properties of diazepam after i.p. injection

Diazepam had a tendency to decrease the global score of anxiety in comparison with a vehicle injection (−60%, $p = 0.0664$; Figure 4). Animals in the Dzp/NAN-190 group had a significantly lower global score of anxiety compared to the Control group (−68%, $p = 0.0162$) and no difference was observed with the Dzp group (−20%, $p = 0.9591$). No difference was observed between the Dzp/Bic and the Control group (−26%, $p = 0.6431$).

4. Discussion

Anxiolytic-like properties of both CH (15 mg/kg, oral administration) and α-CZP (0.8 mg/kg, *i.p.*) were confirmed in the defensive burying test in a rat model which is consistent with previous results using this model (Miclo *et al*, 2001; Violle *et al*, 2006). The results showed that the anxiolytic-like action of CH is probably not dependent of the vagus nerve. The mode of action of both CH and α-CZP involved 5-HT$_{1A}$ receptor as their anxiolytic-like properties

were reversed by the NAN-190, an arylpiperazine derivative that has high affinity for 5-HT_{1A} receptor. On the other hand, bicuculline is a GABA antagonist, which binds directly to the site of this neurotransmitter present on the $GABA_A$ receptor (Ueno *et al*, 1997). The effect of bicuculline on CH and α-CZP anxiolytic-like activity is unclear and only the mode of action of α-CZP seems to involve these receptors.

Vagotomy in comparison to sham operated rats did not abolish the effect of orally administered CH on the global score of anxiety in the conditioned defensive burying test. Thus, the anxiolytic-like properties of CH are not mediated by the vagus nerve and it can be hypothesised that the peptide(s) carrying the bioactivity inside CH may reach its (their) target(s) at a level that is not the intestinal one. Of the different peptides that compose the CH, it has been shown that, at least, the decapeptide α-CZP (sequence 91-100 of the bovine α_{s1}-casein), is a carrier of the CH anxiolytic-like activity (Miclo *et al*, 2001). *In vitro* digestibility of this peptide has already been assessed and the fragment corresponding to the sequence 91-97 was found in significant amount in the hydrolysis medium and was shown to possess comparable anxiolytic-like properties as α-CZP (Cakir-Kiefer *et al*, 2011b). Besides, it was stated that the peptide bonds in the N-terminal region (91-95) of α-CZP are notably resistant to different proteases (pepsin A, chymotrypsin, trypsin and Corolase® PP). Finally, it has been reported that α-CZP transport across Caco-2 cells is facilitated in the presence of bile salts and led to a higher formation of the 91-97 fragment (Cakir-Kiefer *et al*, 2011a). These results agree with an action of one or more peptides of the CH *via* the blood stream and the peptide 91-97 could be a candidate as carrier of the anxiolytic activity after oral administration of CH or α-CZP.

It has been shown that 5-HT_{1A} receptor agonists exhibit anxiolytic-like and antidepressant-like effects (Matsuda *et al*, 1995) and that 5-HT_{1A} receptor-knockout mice showed increased

anxiety-like behaviour (Parks *et al*, 1998; Strauss *et al*, 2013). In the present study the 5-HT_{1A} selective antagonist NAN-190 (Glennon *et al*, 1988) prevented the anxiolytic-like properties of both CH and α-CZP whereas the anxiolytic properties of diazepam were not reversed by NAN-190, which was not described before. This thereby suggests that the anxiolytic-like activity of CH and α-CZP, in contrast to Dzp, involves 5-HT_{1A} receptor in a way that is not yet known. As NAN-190 has been recently demonstrated to also block α_2-adrenergic receptors in submucosal neurons of guinea pigs (Foong and Bornstein, 2009), it could also hypothesised that both CH and α-CZP interact, directly or indirectly, with the adrenergic system. Further experiments using either another 5-HT_{1A} antagonist (e.g. WAY100135 (Fletcher *et al*, 1993)) or a selective α_2-adrenergic receptor (e.g. BRL-44408 (Alberts, 1993)) can then be interesting to carry on with.

The role of $GABA_A$ receptors in CH and α-CZP anxiolytic properties is less clear. Bicuculline, an antagonist of GABA on $GABA_A$ receptors which are the therapeutic target of benzodiazepine (Stahl, 2002), reversed the anxiolytic properties of Dzp, increased anxiety in rats administered with α-CZP despite no significant difference compared to α-CZP administered alone, and did not affect the global score of anxiety of rats administered with CH. $GABA_A$ receptors are composed of 5 subunits and show different combinations of subunits associated with different localisations in the brain as well as different properties. The α_2 subunit seem to be the implicated in the anxiolytic properties of these receptors (Löw *et al*, 2000). As the hydrolysate does not have the side effects of the benzodiazepines, α-CZP, or a shorter derived peptide such as YLGYLEQ, might bind only to a specific population of $GABA_A$ receptor subtypes and this could explain the lower affinity of this peptide for $GABA_A$ receptor that was determined by competition with tritiated flunitrazepam (10,000 times lower than affinity of Dzp) (Miclo *et al*, 2001). Flunitrazepam is a non-selective benzodiazepine, which bind to $GABA_A$ receptors with,

in addition to β and γ subunits, an α_1, α_2, α_3 or α_5 subunit (You *et al*, 2010). In this study, the inhibition of the $GABA_A$ receptors by bicuculline only had a tendency to counteract α-CZP anxiolytic-like properties. This lower impact of an inhibition of the $GABA_A$ receptors observed might be consistent with a selectivity of $GABA_A$ receptor subtypes by α-CZP since bicuculline-insensitive GABA receptors were discovered (Johnston, 2013).

Previous work on anxiolytic peptides derived from bovine α_{s1}-casein showed that after an oral administration of α-CZP in mice, the anxiolytic-like properties of this peptide (in an elevated plus-maze model) were blocked by the inhibition of 5-HT_{1A}, dopamine D_1 or $GABA_A$ receptors (Mizushige *et al*, 2013b). A smaller peptide, YL, that corresponds to fragment 91-92 and 94-95 of bovine α_{s1}-casein, but that may be also released from numerous proteins since this sequence frequency is about 1.34% (Vonderviszt *et al*, 1986) also possessed anxiolytic-like properties, which were mediated by 5-HT_{1A}, dopamine D_1 and $GABA_A$ receptors despite having no affinity for any of these receptors and this dipeptide could trigger the different receptors in the following order: 5-HT_{1A}, dopamine D_1 and eventually $GABA_A$ receptor (Kanegawa *et al*, 2010). Dipeptide YL was not found after *in vitro* digestion of α-CZP by Corolase® PP (Cakir-Kiefer *et al*, 2011b) and was present at only 0.03% after digestion of bovine α_{s1}-casein (Mizushige *et al*, 2013b).

The observed differences between the results obtained with CH, α-CZP and Dzp may implicate a different mode of action. Differences related to 5-HT_{1A} receptor pathways and the specific binding of α-CZP on $GABA_A$ receptor subtypes which are involved in anxiolysis (α_2-containing $GABA_A$ receptors) (Morris *et al*, 2006), but not to those involved in amnesia, addiction or sedation (α_1-containing $GABA_A$ receptors) (Rudolph *et al*, 1999) could explain the absence of side effects for CH which are traditionally associated with benzodiazepines (memory-

297 impairing, tolerance or dependence) (Messaoudi *et al*, 2009). In addition, the observation that
298 CH and α-CZP exhibit very similar pharmacological profiles with very small differences may
299 be explained by their mode of administration as CH was administered orally while α-CZP was
300 injected intraperitoneally. The amounts of administered α-CZP are also different since the oral
301 dose of peptide administered via the hydrolysate was 0.28 mg/kg whereas that of pure peptide
302 by *i.p.* injection was 0.8 mg/kg. The higher dose of α-CZP after an *i.p.* injection can have
303 revealed the affinity of the peptide for $GABA_A$ receptors that could not be detected with the
304 lower dose which was orally administered. Moreover, after oral administration, peptides in CH
305 (including α-CZP) undergo hydrolysis by proteases of the gastrointestinal tract and a slight
306 differences in modes of action can result of this. Other peptides present in CH may also interfere
307 with α-CZP and modulate its anxiolytic-like properties, thus explaining also the slight
308 differences between CH and α-CZP modes of action.
309

310 **Author contributions**

311 Study concept and design: N.V., J.S. Acquisition of data: N.V. Analysis of data: S.B., N.V.
312 Interpretation of data: S.B., L.M., N.V. Drafting the manual: S.B. Critical revision of the
313 manuscript for important intellectual content: C.C., C.C.-K., D.T., L.M., N.V., J.S.
314

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Conflict of interest

J.S. works at Ingredia, SA, Arras

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Table 1. Summary of the tested products, used doses, ways of administration, time of administration before testing and groups names of the experiment 2.

Test product		**Antagonist**			
Dose, administration	*Time before test*	*Dose, administration*	*Time before test*	**Group name**	***n***
Tryptic casein hydrolysate (CH)					
Vehicle (3 mL/kg, *per os*)	60 min	Vehicle (2 mL/kg, *i.p.*)	65 min	Control	12
Diazepam (3 mg/kg, *per os*)	60 min	Vehicle (2 mL/kg, *i.p.*)	65 min	Dzp	12
Casein hydrolysate (15 mg/kg, *per os*)	60 min	Vehicle (2 mL/kg, *i.p.*)	65 min	CH	12
Casein hydrolysate (15 mg/kg, *per os*)	60 min	NAN-190 (3 mg/kg, *i.p.*)	65 min	CH/NAN-190	12
Casein hydrolysate (15 mg/kg, *per os*)	60 min	Bicuculline (3 mg/kg, *i.p.*)	65 min	CH/Bic	12
α-casozepine					
Vehicle (2 mL/kg, *i.p.*)	30 min	Vehicle (2 mL/kg, *i.p.*)	45 min	Control	12
Diazepam (1 mg/kg, *i.p.*)	30 min	Vehicle (2 mL/kg, *i.p.*)	45 min	Dzp	12
α-casozepine (0.8 mg/kg, *i.p.*)	30 min	Vehicle (2 mL/kg, *i.p.*)	45 min	α-CZP	12
α-casozepine (0.8 mg/kg, *i.p.*)	30 min	NAN-190 (1 mg/kg, *i.p.*)	45 min	α-CZP/NAN-190	12
α-casozepine (0.8 mg/kg, *i.p.*)	30 min	Bicuculline (1 mg/kg, *i.p.*)	45 min	α-CZP/Bic	12

		Diazepam			
Vehicle (2 mL/kg, *i.p.*)	30 min	Vehicle (2 mL/kg, *i.p.*)	45 min	Control	8
Diazepam (1 mg/kg, *i.p.*)	30 min	Vehicle (2 mL/kg, *i.p.*)	45 min	Dzp	8
Diazepam (1 mg/kg, *i.p.*)	30 min	NAN-190 (1 mg/kg, *i.p.*)	45 min	Dzp/NAN-190	8
Diazepam (1 mg/kg, *i.p.*)	30 min	Bicuculline (1 mg/kg, *i.p.*)	45 min	Dzp/Bic	8

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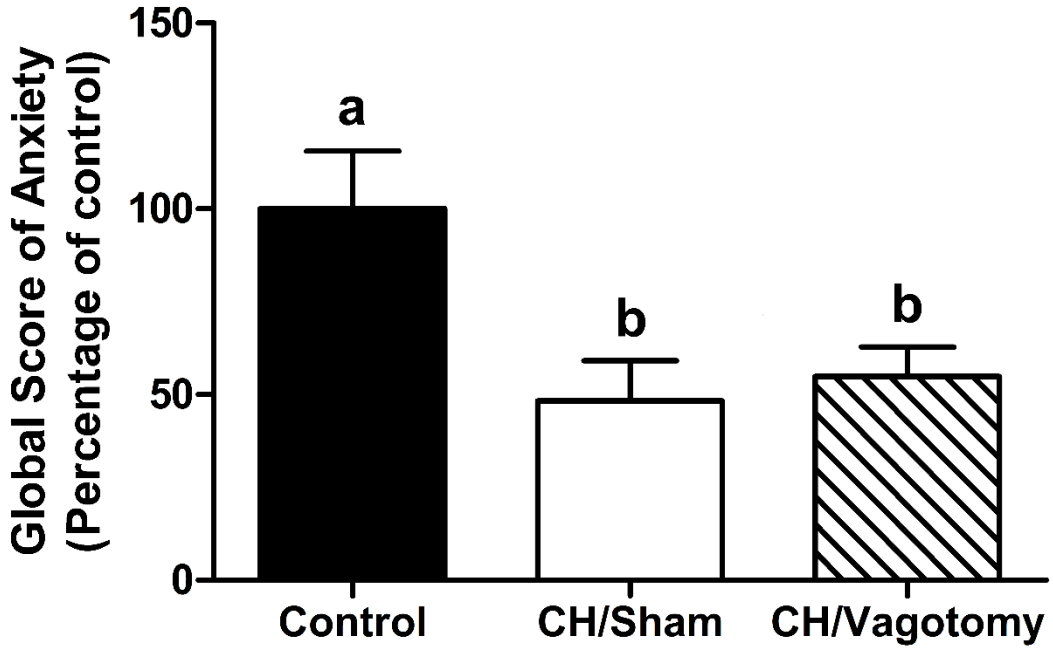
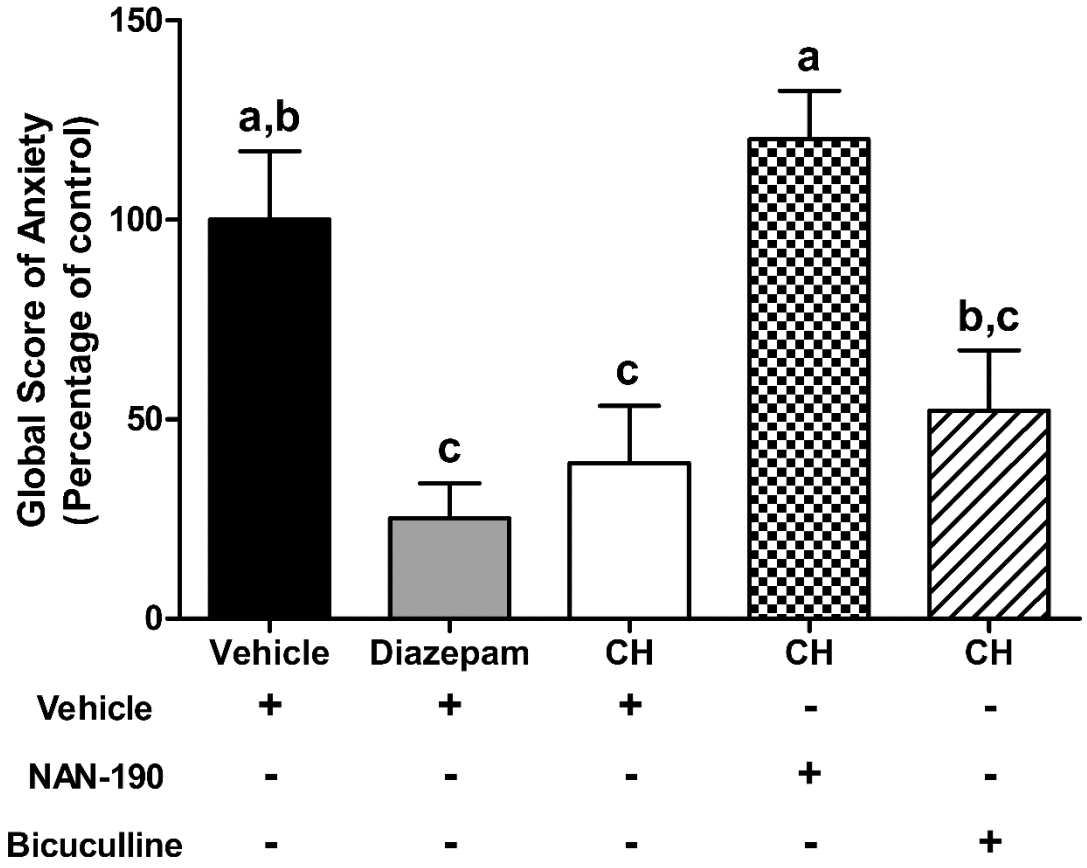


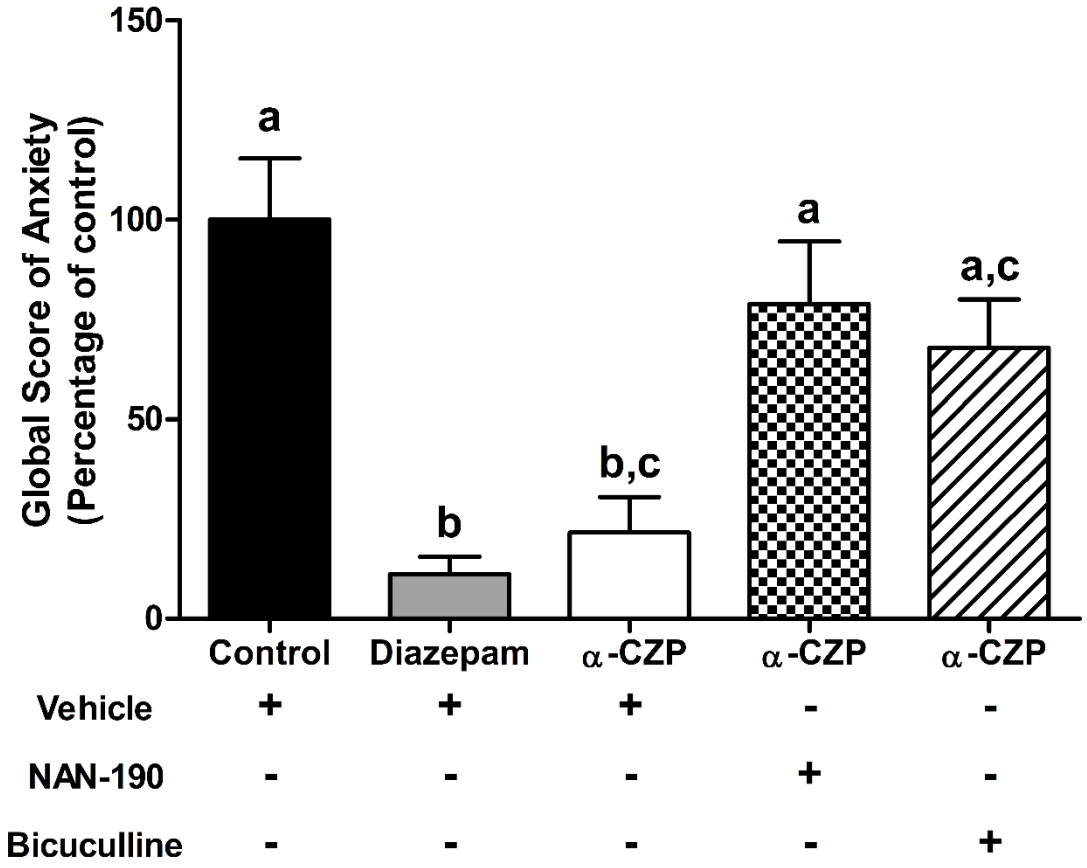
435

436 **Figure 1.** Effects of CH, orally administered at a single dose of 15 mg/kg, on the global score
437 of anxiety in a conditioned defensive burying model in the vagotomised male Wistar rat
438 (*n* = 8). Data are mean ± SEM. Means with different letters are significantly different (Tukey
439 post-hoc, $p < 0.05$).



440

441 **Figure 2.** Effects of NAN-190 (3 mg/kg, *i.p.*) and bicuculline (3 mg/kg, *i.p.*) on CH (15 mg/kg,
442 oral) anxiolytic action in a conditioned defensive burying model in rats (n = 12). Data are
443 mean ± SEM. Means with different letters are significantly different (Tukey post-hoc,
444 $p < 0.05$).



445

446 **Figure 3.** Effects of NAN-190 (1 mg/kg, *i.p.*) and bicuculline (1 mg/kg, *i.p.*) on α-CZP

447 (0.8 mg/kg, *i.p.*) anxiolytic action in a conditioned defensive burying model in rats (n = 12).

448 Data are mean ± SEM. Means with different letters are significantly different (Tukey post-hoc,

449 $p < 0.05$).

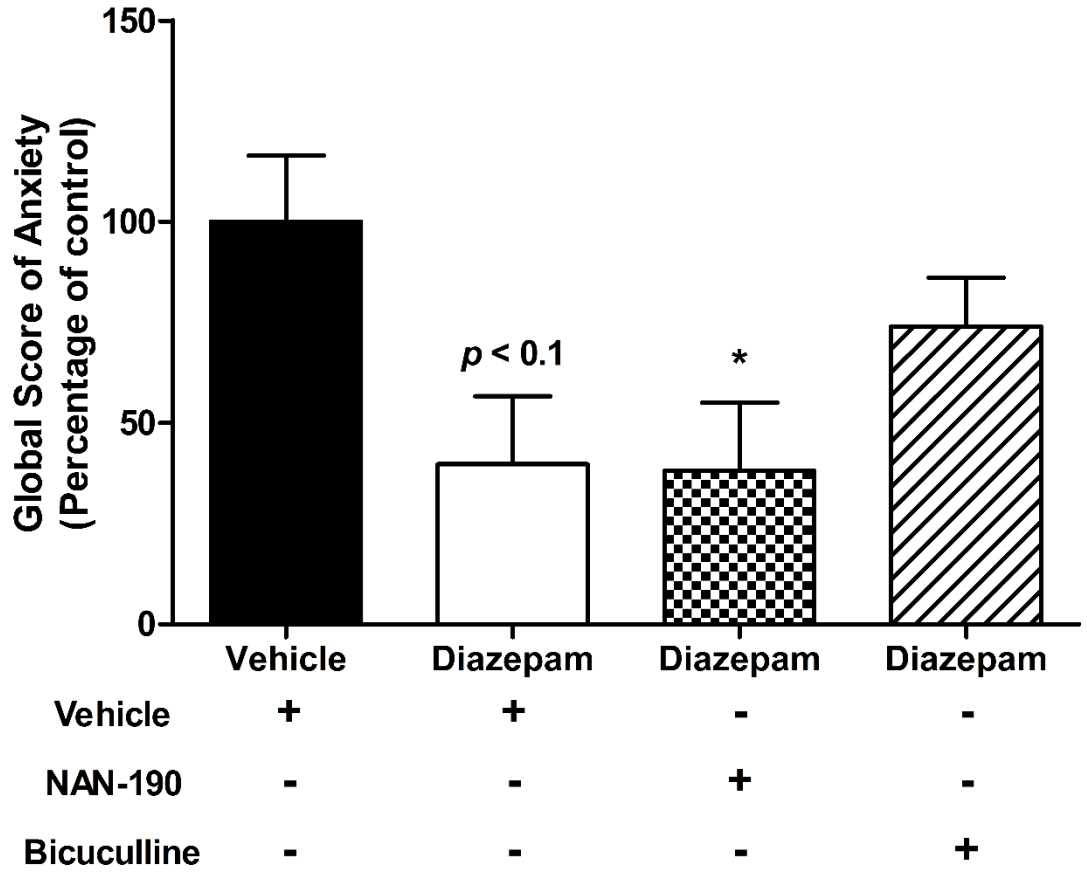


Figure 4. Effects of NAN-190 (1 mg/kg, *i.p.*) and bicuculline (1 mg/kg, *i.p.*) on diazepam (1 mg/kg, *i.p.*) anxiolytic action in a conditioned defensive burying model in rats (n = 8). Data are mean ± SEM. *p < 0.05 compared to the Vehicle group (Tukey post-hoc).

PART III – GENERAL DISCUSSION AND CONCLUSION

1 DECIPHERING THE CENTRAL MODE OF ACTION OF α-CASOZEPINE

The primary objective of this thesis was to evaluate whether α-casozepine (α-CZP), an anxiolytic decapeptide derived from bovine α_{s1}-casein, had an effect on the central nervous system, and more specifically in the brain regions involved in anxiety regulation. As previously suspected for the industrial tryptic hydrolysate of α_{s1}-casein, the first study revealed that changes in neuronal activity were only detected in animals subjected to an anxiety-inducing situation. Both studies also pointed out that the changes observed after an intraperitoneal (i.p.) injection of α-CZP were not the same as after an injection of diazepam, a reference benzodiazepine, or more importantly as after an injection of a shorter peptide derived from α-CZP. This section reviews these different results and raises some questions concerning the central mode of action of α-CZP.

1.1 Central effects of α-casozepine

The results showed that an i.p. injection of α-CZP in an anxiety-inducing situation had an impact on the activity of central circuits implicated in anxiety regulation in the mouse brain (***FIGURE 1.1***). The modulation of c-Fos expression differed between the different brain regions that were studied. More specifically, the i.p. injection of α-CZP decreased neuronal activity in the accumbens nucleus, the hippocampal formation, the bed nucleus of the stria terminalis (BNST), as well as the paraventricular, the dorsomedial, and the ventromedial nuclei of the hypothalamus. In contrast, the i.p. injection of α-CZP raised neuronal activity in most nuclei of the amygdala as well as in the raphe magnus nucleus. In addition, α-CZP did not impact neuronal activity in the prefrontal cortex, the periaqueductal grey, as well as the nucleus of the tractus solitarius.

This demonstrated that a bioactive peptide derived from food protein had an impact on the central nervous system after an i.p. injection. The impact of other anxiolytic-like compounds derived from natural products such as lavender oil (Shaw *et al*, 2011) or yokukansan (Shoji and Mizoguchi, 2013), a traditional Japanese remedy, on c-Fos expression has already been studied. Both compounds had less impact on neuronal activity than α-CZP, but this may be either due to the protocol, or to the fact that less brain areas were studied.

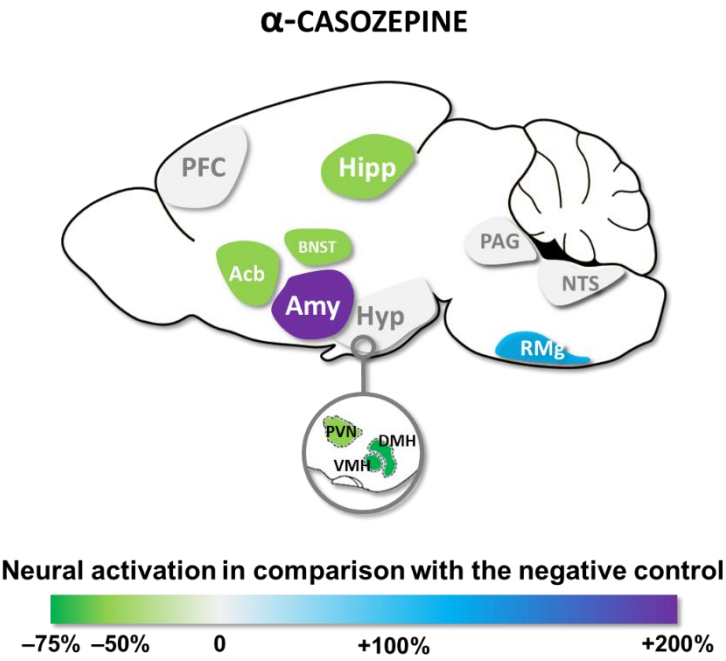


FIGURE 1.1 – Effect of α-CZP on neural activity in an anxiety-inducing situation in comparison with the vehicle.

Acb, accumbens nucleus; Amy, amygdala; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; CeA, central nucleus of the amygdala; Hipp, hippocampal formation; Hyp, hypothalamus; PAG, periaqueductal grey; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; NTS, nucleus of tractus solitarius; RMg, raphe magnus nucleus; VMH, ventromedial nucleus of the hypothalamus.

One possible explanation of the specific action of α-CZP in the modulation of c-Fos expression in the mouse brain is the distribution of some specific $GABA_A$ receptors in the CNS. Indeed, as this peptide displays an affinity for the BZD site of $GABA_A$ receptors with an IC_{50} of 88 µM (Miclo *et al*, 2001), its anxiolytic-like properties can be partly explained by its binding on this site. As tryptic hydrolysate containing α-CZP does not have other pharmacological properties of BZDs such as sedative or memory impairment effects (Messaoudi *et al*, 2009; Dela Peña *et al*, 2016), it could be hypothesised that this peptide has a specific affinity for the α_2-containing $GABA_A$ receptors, which specifically mediate the anxiolytic properties of BZDs (Möhler, 2012). Interestingly, these α_2-containing $GABA_A$ receptors are highly concentrated in the limbic structures such as the amygdala, the accumbens nucleus or the hippocampal formation (D'Hulst *et al*, 2009), where α-CZP modulates neuronal activity. However, the

chronology by which regions are modulated by the peptide cannot be determined with the experiments conducted in this thesis. An interaction with $GABA_A$ receptor subtypes containing an α_2-subunit still remains as an hypothesis. Indeed, the affinity of the peptide for the BDZ site of the $GABA_A$ receptor was determined in competition with flunitrazepam (Miclo *et al*, 2001), a non-selective benzodiazepine. Flunitrazepam binds to $GABA_A$ receptors containing an α_2-subunit but also to those containing an α_1-, α_3-, or α_5-subunit, and even to receptors possessing the α_4-subunit that are said insensitive to diazepam (You *et al*, 2010).

1.2 An anxiety-dependent action

This work demonstrated that an anxiety-inducing situation was mandatory to observe changes on neuronal activation after an i.p. injection of α-CZP. This result agree with previous studies showing that CH was more active in anxious individuals. Indeed, a preclinical study demonstrated that the positive effects of CH on sleep in rats was only observed when the rats when submitted to a chronic mild stress (Guesdon *et al*, 2006). Palestrini and colleagues showed that in Beagle dogs the consumption of a food enriched with CH did not alter the profile of non-anxious animals while it decreased the level of anxiety in anxious animals (Palestrini *et al*, 2010). After 59 days of this diet, the anxiety level of the initially anxious dogs was the same as that of non-anxious animals. In the same way, the positive effects of CH on several stress-associated symptoms after a 30 days supplementation in women was specific for women with high intensities symptoms at the beginning of the treatment (Kim *et al*, 2007).

These results seem to contradict other studies that were not specifically conducted on anxious populations but that still demonstrated an anxiolytic-like effect of either α-CZP amongst rodents (Miclo *et al*, 2001) or CH in clinical studies (Messaoudi *et al*, 2005). This lies in the specificity of the protocols used to study the anxiolytic-like effects of these molecules. Indeed, the evaluation of anxiety levels amongst rodents consists of placing them in an anxiety-inducing situation, thus raising momentarily the state-anxiety levels but not the trait-anxiety ones. This is quite the same with clinical studies, as both situations (the Stroop test and the cold pressor test) raising the state-anxiety levels of the individuals. As both state-anxiety and trait-anxiety participate to the anxiety levels that can be measured by the observer, it is then easy not to be able to distinguish them (Spielberger, 1966).

The present results revealed that the light/dark box, the device we used to trigger the anxiety-inducing situation, raised neuronal activity in several regions implicated in anxiety regulation such as the prefrontal cortex, the hippocampal formation, the accumbens nucleus, the BNST and some nuclei of the hypothalamus (paraventricular, dorsomedial and ventromedial nuclei) (***FIGURE 1.2***). These results agree with previous observations on the impact of other ethological models of anxiety on neuronal activity such as the elevated plus-maze, which is also based on the aversion of rodents for wide bright areas (Duncan *et al*, 1996; Kovács, 1998). The increase of neuronal activity in these regions may explain part of the necessity of an anxiety-inducing situation to observe α-CZP anxiolytic-like properties. Indeed, the peptide may not be able to down-regulate neuronal activity if the regions where it is supposed to act are not activated by an anxiety-inducing situation.

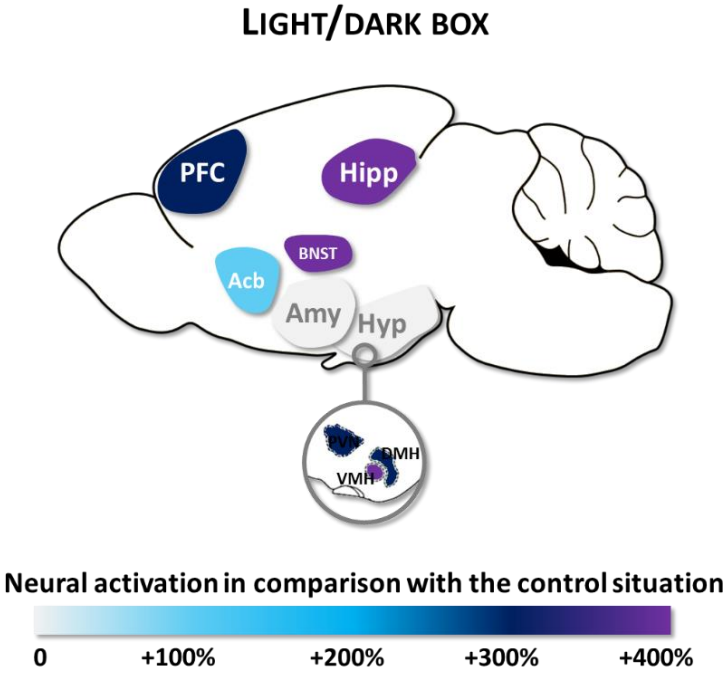


FIGURE 1.2 – Effect of LDB on neural activity in comparison with a non anxiety-inducing situation.

Acb, accumbens nucleus; Amy, amygdala; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; Hipp, hippocampal formation; Hyp, hypothalamus; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus.

It has also been demonstrated that stress and psychological disorders, such as anxiety disorders or depression, induce intestinal physiological changes (Gareau *et al*, 2008; Julio-Pieper *et al*, 2014; O'Malley *et al*, 2010). These changes may increase the permeability of the intestinal barrier in stress conditions and lead to an increased absorption of the peptides of CH, including α-CZP, after an oral administration.

1.3 Is α-casozepine a benzodiazepine-mimetic peptide?

α-CZP shared some similarities with diazepam action on their impact on neural activity as they both decrease the activity of the accumbens nucleus, the hippocampal formation and some nuclei of the hypothalamus (paraventricular, dorsomedial and ventromedial nuclei) compared to the vehicle in an anxiety-inducing situation (***FIGURE 1.3***). However, numerous differences exist between the two molecules in terms of neuron activity modulation. Indeed, α-CZP but not diazepam also decreased the activity of the BNST while it increased the activity of some nuclei of the amygdala and of the raphe nucleus compared to the vehicle in the same conditions. On the other hand, diazepam decreased activity in the prefrontal cortex and its effect in the hippocampal formation is greater than that of α-CZP, while it increased the activity of the central nucleus of the amygdala and of the NTS compared to the vehicle (***FIGURE 1.3***).

These differences question the benzodiazepine-like nature of α-CZP a peptide. The initial identification of α-CZP was performed by a screening of the different peptides of CH on BZD binding sites, via a radiolabelled ligand assay using methyl-[^{3}H]-flunitrazepam (Miclo *et al*, 2001). As this peptide was also shown to carry anxiolytic-like properties in rodents (Miclo *et al*, 2001), it appeared questionable that a peptide with anxiolytic-like properties was identified via the wrong receptor screening test. Moreover, the tryptic hydrolysate containing α-CZP has been shown to reduce the epileptic symptoms caused by pentylenetetrazole in rats (Miclo *et al*, 2001) while its *in vitro* action on chloride ion influx was blocked by bicuculline, a $GABA_A$ receptor antagonist (Dela Peña *et al*, 2016), making them arguments in favour of a benzodiazepine-mimetic hypothesis. Two hypotheses can then be articulated to explain the differences observed between α-CZP and diazepam regarding on their effects on neuronal activity.

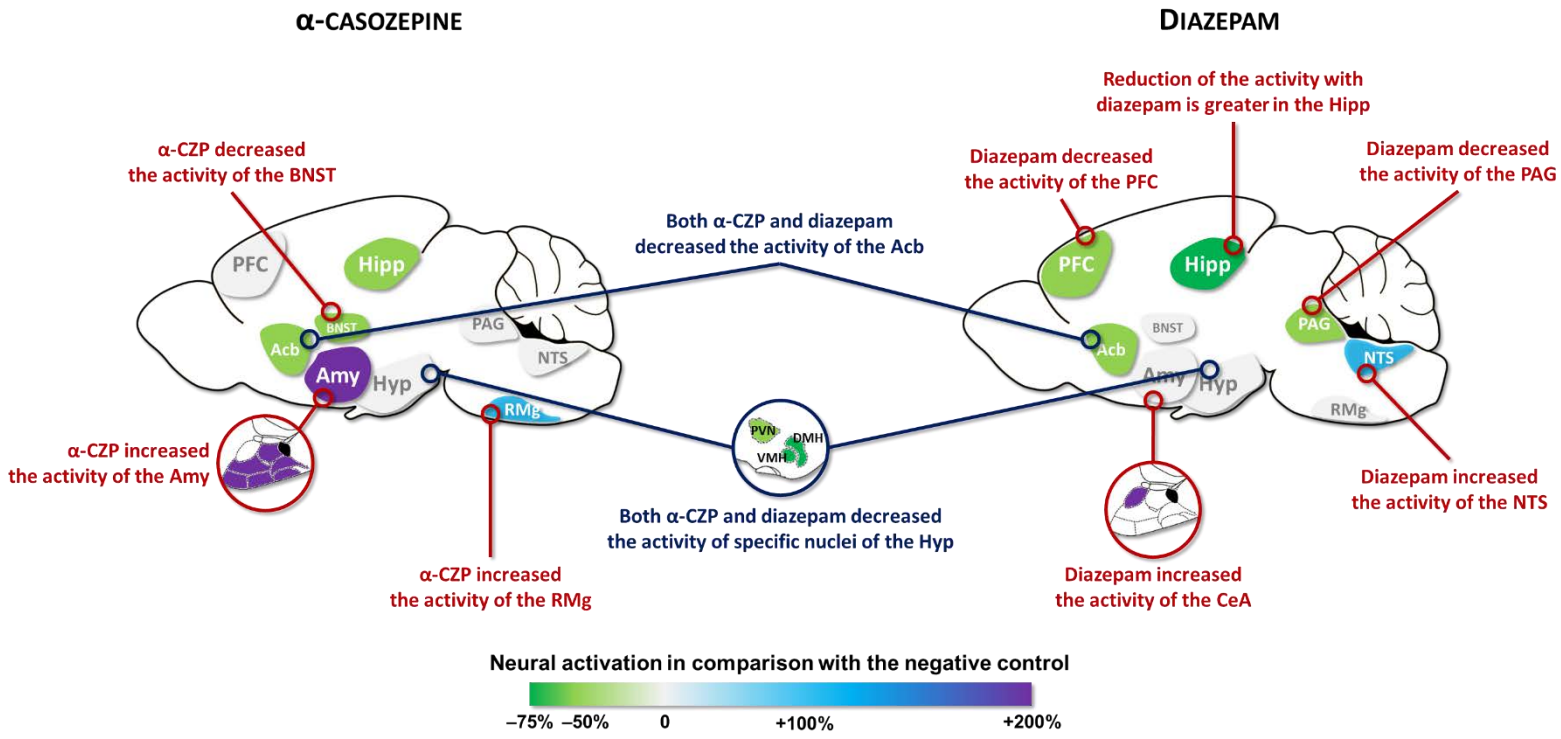


FIGURE 1.3 – Activities of α-casozepine and diazepam: similarities and differences on neural activation in an anxiety-inducing situation.

Acb, accumbens nucleus; Amy, amygdala; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; CeA, central nucleus of the amygdala; Hipp, hippocampal formation; Hyp, hypothalamus; PAG, periaqueductal grey; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; NTS, nucleus of tractus solitarius; RMg, raphe magnus nucleus; VMH, ventromedial nucleus of the hypothalamus. Similarities are in blue, differences in red.

Assuming that α-CZP is a BZD-like peptide, it has been since then demonstrated that specific $GABA_A$ subtypes mediate different central effects of BZDs including sedation, anxiolysis, or hypnosis (Nutt, 2006). As α-CZP do not trigger sedative effects as well as other side effects associated with BZD consumption (Messaoudi *et al*, 2009; Dela Peña *et al*, 2016), **it could be hypothesised that α-CZP has a specific affinity for receptors mediating only the anxiolytic properties of BZD**: α_2-containing $GABA_A$ receptors (Möhler, 2012). High concentrations of these receptors have been measured in the limbic structures such as the amygdala, the accumbens nucleus or the hippocampal formation (D'Hulst *et al*, 2009), where α-CZP modulates neuronal activity, as seen above. On the other hand, the α_1-containing $GABA_A$ receptors, mediating the sedative effects of BZDs, are concentrated in the cerebellum, the thalamus, and the cerebral cortices, and notably in the prefrontal cortex where α-CZP have no effect on neuronal activity. Moreover, the α_2-containing $GABA_A$ receptors account for only 20% of the total $GABA_A$ receptors (McKernan and Whiting, 1996), maybe explaining in part the affinity of α-CZP for the BZD fixation site on $GABA_A$ receptors which is 10,000 times lower than that of diazepam (Miclo *et al*, 2001).

Another hypothesis is that **α-CZP most likely involve other neurotransmitters systems and more particularly the serotoninergic system**. Indeed, α-CZP increased the activity of raphe nucleus, which gathers the majority of serotoninergic neurons of the brain (Millan, 2003). Moreover, it seems that the anxiolytic-like properties of CH relied also on serotoninergic receptors type 1A (5-HT_{1A}) while those of diazepam did not (unpublished results), reinforcing the serotoninergic hypothesis. α-CZP could then also been brought closer to another anxiolytic, the buspirone. Besides implication of 5-HT_{1A} receptors in its mode of action (Peroutka, 1988), this anxiolytic drug share with α-CZP an absence of sedative effects traditionally (Seidel *et al*, 1985), and muscle relaxant and anticonvulsant effects (Riblet *et al*, 1984) associated with BZDs. Moreover, it does not induce cognitive impairment (Erwin *et al*, 1986), tolerance, dependence or withdrawal effects as well as it does not interact with alcohol (Argyropoulos *et al*, 2000) unlike BZDs. Eventually, its effects are observed after two to three weeks of treatment in humans (Lader and Olajide, 1987), which can be compared to the effects of α-CZP (Kim *et al*, 2007). It could then be hypothesised that α-CZP relies on BZDs binding sites for a short-term effect, and then on the serotoninergic system to trigger more perennial anxiolytic properties.

In addition, α-CZP displays some amino acid sequence analogies with **opiate peptides** already identified in milk proteins (Zioudrou *et al*, 1979). Indeed, the N-terminal motif of the YLGYLEQLLR sequence of α-CZP is close to the RYLYL and RYLYLE sequences of opiates peptides identified from the bovine α_{s1}-casein sequence (Loukas *et al*, 1983). The pattern consisting of a tyrosine in the N-terminal end associated with an aromatic amino acid in the 3rd or the 4th position has been proven to be crucial to carry the opiate effects of the peptide (Chang *et al*, 1981). As α-CZP contains this pattern, it can also be hypothesised **that the opioid system** mediates part of the anxiolytic-like properties of the peptide. α-CZP can either have an affinity for opiate receptors such as RYLGYL and RYLGYLE, or trigger a central pathway involving the opioid system, such as diazepam, going in the 'benzodiazepine-mimetic' direction (Randall-Thompson *et al*, 2010). The delta and mu receptors can be candidates to both hypotheses (Le Merrer *et al*, 2006; Perrine *et al*, 2006). Nevertheless, peptides with an arginine residue in amino-terminal position display opioid activity in the models used by the authors whereas the corresponding peptides lacking this residue (YLGYL and YLGYLE) are almost inactive in these models (Loukas *et al*, 1983). Until now, a longer peptide extended in its carboxy-terminal part and corresponding to α-CZP has never been tested.

1.4 Do peptides derived from α-casozepine, as YLGYL, act differently?

It has been previously shown that YLGYLEQ was a major breakdown product after hydrolysis of α-CZP in *in vitro* digestion conditions (Cakir-Kiefer *et al*, 2011b). This peptide displays anxiolytic-like properties in behavioural models using rats. YLGYL was a second major product of hydrolysis in the same experimental conditions (Cakir-Kiefer *et al*, 2011b). In the present work, we have shown that this peptide also exhibits anxiolytic-like properties in mice. Nevertheless, YLGYL displayed some differences with α-CZP in terms of neuronal activity modulation compared to the vehicle in an anxiety-inducing situation (***FIGURE 1.4***). Indeed, while α-CZP increased c-Fos expression in all the structures of the amygdala, YLGYL, only increased neuronal activity in the cortical nuclei, compared to the vehicle. On the other hand, the increased of c-Fos expression in the raphe magnus nucleus was greater after YLGYL i.p. injection than after α-CZP injection, compared to the vehicle.

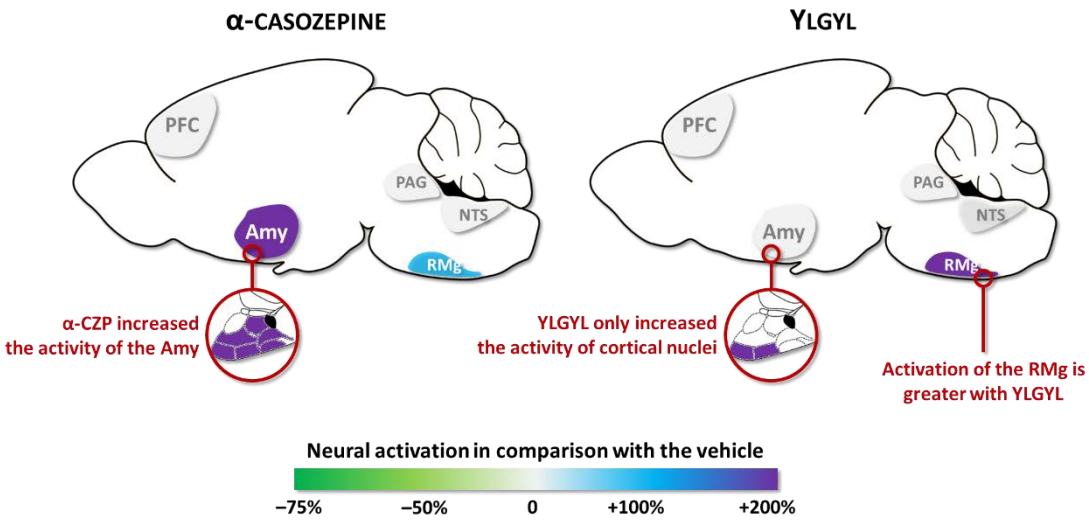


FIGURE 1.4 – α-casozepine and YLGYL differences on neural activation after a anxiety-induced situation.

Amy, amygdala; PAG, periaqueductal grey; PFC, prefrontal cortex; NTS, nucleus of tractus solitarius; RMg, raphe magnus nucleus.

This raises the question whether α-CZP and its derivative YLGYL would centrally act the same way to trigger their anxiolytic-like properties. It is at this step difficult to answer this question and further experiments involving either *in vivo* or *in vitro* pharmacological techniques are required to further explore these mechanisms. Thus, the two peptides may have different affinities for specific neurotransmitters receptors. As YLGYL is close to RYLGYL, an opiate peptide (Loukas *et al*, 1983), this pathway could be favoured, although these authors described YLGYL as almost inactive in the experimental models of opiate activity that they used.

The anxiolytic activity of α-CZP could be a very complex process if this peptide and its derived peptides modulate the neuronal activity in different ways. In addition, the peptides YL and YLG that correspond to fragments of the peptide also showed an anxiolytic profile but the proposed metabolic cascade that could lead to biological activity does not involve the benzodiazepine site of GABAA receptor (Kanegawa *et al*, 2010). The questions of which peptide(s) is (are) most likely to cross physiological barriers and which peptide(s) reaches (reach) the area(s) triggering the anxiolytic activity remain unclear.

1.5 The limits of c-Fos studies

c-Fos studies hold several advantages. Firstly, they allow the analysis of a distributed network via the study of the whole brain, while neuronal activity can be studied up to the cellular resolution. Moreover, as a delayed marker of brain activation, there is no interference with other manipulation close to the animal death (such as the injection of sodium pentobarbital). Finally, it already exists a wide literature on c-Fos studies, as the most studied neuronal activity marker, which allows multiple comparisons against the accessible scientific database.

However, despite its usefulness to measure neuronal activation, the evaluation of neuronal activity via c-Fos has several limitations. The first one is that **c-Fos is not a direct measure of the neuronal activation**. Despite the number of studies linking c-Fos expression and neuronal depolarisation, it is important to remind that c-Fos expression is induced via the signal transduction pathway and not by the depolarisation *per se* (Hoffman and Lyo, 2002). A possible way to evaluate direct neuronal activation would be to use electrophysiology. This technique directly measure neuron firing in *in vivo* models of anaesthetised rodents submitted to different pharmacological treatments to mimic anxiety or its associated treatments, such as benzodiazepines (Crespi, 2009). However, it is harder to record several regions at the same time and it must then be repeated to evaluate different neurons from different brain areas. The use of electroencephalography or magnetoencephalography can help to resolve the study of the whole brain. However, the spatial resolution is less interesting than electrophysiology, and it is more used to evaluate changes of brain activity over time coupled with specific actions (such as activity versus sleep) than to evaluate the specific role of some brain regions in an action, with an exception for the hippocampal formation (Siok *et al*, 2012). Functional magnetic resonance imaging (fMRI) in rodent is still hard to implement due to the low spatial resolution in small animals.

As a downside of its delayed neuronal marker, **c-Fos suffers from a low time resolution**, as the expression of the protein is evaluated around 90 minutes after the stimulus. To decrease the bias set by the protocol conditions, the studied stimulus must be the only thing changing between groups to limit parasite stimuli. Moreover, repetitive stimulation of animals is bound to decrease c-Fos signal. As a direct conclusion, some authors consider c-Fos as a learning marker as well as a neuronal marker (Chung, 2015).

It has also been demonstrated **that some neurons do not trigger c-Fos expression** when stimulated while others always express the protein (Hoffman and Lyo, 2002). Moreover, **the specific chemical nature of the activated neurons cannot be determined** via this technique, and double labelling methods targeting neurotransmitters must then be implemented. Eventually, as a post-mortem technique**, animals cannot be repeatedly tested** which may impact the results if the stimulus is not well chosen.

To compensate the limits of c-Fos, other transcription factor acting as neuronal activity marker can be used such as Early growth response protein 1 (Egr1) or phosphorylated cAMP response element-binding protein (pCREB). Egr1 is similar to c-Fos as it is also a transcription factor. However, its basal expression level is higher than that of c-Fos and temporal profile induction is different, c-Fos being transient while Egr1 is more sustained (Zangenehpour and Chaudhuri, 2002). On the other hand, pCREB is related to the formation of memory and is linked to c-Fos expression (Hoffman and Lyo, 2002; Silva *et al*, 1998).

2 CONCLUSION AND PERSECTIVES

This work showed that (i) an anxiety-inducing situation is mandatory to trigger the anxiolytic-like properties of α-CZP; (ii) a bioactive peptide coming from food and i.p. injected impact brain activation in specific regions involved in anxiety regulation; (iii) the activity patterns triggered by α-CZP injection are different from those triggered by an i.p. injection of diazepam, a reference benzodiazepine, at the same concentration; and (iv) a shorter peptide coming from the *in vitro* digestion of α-CZP and also displaying anxiolytic-like properties, does not modulate brain activity exactly the same as α-CZP.

This work contributes to the understanding of the central mode of action of α-CZP, and on the mode of action of the peptide (***FIGURE 2.1***). Several questions have been raised and could stimulate future research on this peptide:

Does the peptide act in the brain? Despite influencing the CNS activity, it has not yet been proven that the peptide act directly *in* the brain as the changes observed can be due to an indirect peripheral activation of another mediator. An intracerebroventricular injection of the peptide could then partly answer that question (it cannot be pushed aside that the peptide may work in that way but that after an oral administration, a mediator might be involved to trigger the anxiolytic-like properties of α-CZP). Nevertheless, it has been shown that the peptide is able of modulating the neuronal activity without being hydrolysed by proteases or peptidases of the digestive tract to induce the formation of an active peptide since the intraperitoneal injection eliminates the contribution of digestive tract.

Reasoning as a dietary supplement. As the peptide is to be given as a dietary supplement within CH, carrying on the effects of an oral administration of the peptide on neuronal activation needs to be considered as the difference of the way of administration may impact central activity. As a dietary supplement the peptide is also more likely to be consumed more than once. Comparison between an acute and a chronic administration can also reveal some differences between the two types of administration.

Which receptors and neurotransmitters systems are involved in its mode of action? As some brain structures involved in the mode of action of α-CZP have been identified and as there are pharmacological studies, identifying the neurotransmitters involved in the mechanism of action of α-CZP, the next step would be to combine both information for a more acute understanding of the mechanisms. The injection of specific antagonist in specific brain structures, using stereotaxic surgery, could be a way to answer this question. As serotoninergic and GABAergic systems have already been identified, it would then be interesting to start with them and for instance in the amygdala. The specific affinity of α-CZP on α_2-containing $GABA_A$ receptors could also be tested with KO models or specific antagonist of α_2-containing $GABA_A$ receptors. As the peptide also display some similarities with opiate peptides, the affinity of α-CZP for opiate receptors *in vitro*, as well as the involvement of opiate receptors in the *in vivo* effects of α-CZP should also be evaluated. Another way would be to use co-localisation techniques to identify the chemical nature of activated neurons *via* immunofluorescence.

Is it the same for humans? The possible effect(s) of the administration of α-CZP in human brain activity should also be addressed. Indeed, as α-CZP is used as a dietary supplement for humans with anxiety issues, clinical studies need to be undertaken to confirm the results on brain activity in humans. As the development of the prefrontal cortex region is different between humans and rodents, cortex being less developed in rodents than humans, and as α-CZP has been proven not to impact the activity in this region, some differences may be expected between the modulation of brain activity by α-CZP between humans and rodents. The use of techniques such as electroencephalogram as a first step or fMRI as a second step could help to answer that question. Placing individuals in an anxiety situation, such as the Stroop test, where CH has proven to be efficient, could mimic the anxiety-inducing situation introduced in the present work.

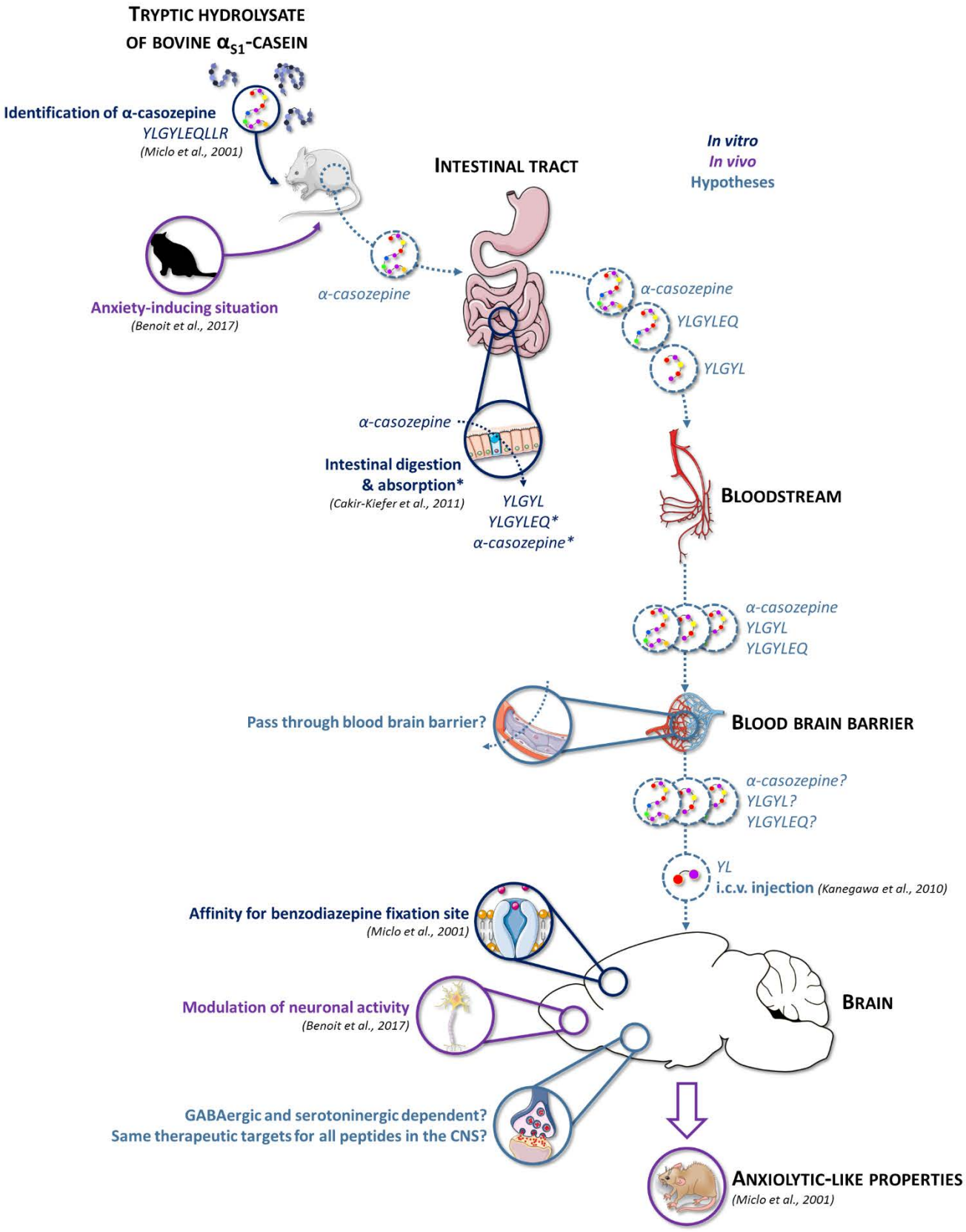


FIGURE 2.1 – Understanding the mode of action of α-casozepine *via* rodents' and *in vitro* models.

PART IV – ANNEX

1 RESUME SUBSTANTIEL

1.1 Introduction générale

La découverte de molécules d'origine alimentaire possédant des propriétés biologiques au-delà des simples apports en nutriments pour l'organisme a permis d'ouvrir un nouveau champ d'action pour le développement de molécules d'intérêt pour la santé humaine. Les **peptides bioactifs**, qui sont des fragments protéiques ayant des impacts positifs sur la santé de l'organisme (Kitts and Weiler, 2003; Korhonen, 2009) en font partie. Ces peptides possèdent différents avantages par rapport aux molécules pharmacologiques « classiques » qui leur permettraient d'être utilisés en médecine humaine (Craik *et al*, 2013; Fosgerau and Hoffmann, 2015) : bonne efficacité, meilleure sélectivité, bon profil sécurité, pas d'effet secondaire, plus facile à synthétiser… Le lait de vache, qui contient jusqu'à 35 grammes de protéines par litre, est l'une des sources d'obtention de ces peptides bioactifs (Korhonen, 2009). Parmi les très nombreux peptides issus des protéines laitières, certains ont su montrer des effets au niveau central en visant certains systèmes de neurotransmetteurs (notamment le système opioïdergique avec les β-casomorphines), qui permettrait, entre autres, d'expliquer une partie des effets calmants associés à la consommation de lait de vache (Brezinová and Oswald, 1972; Southwell *et al*, 1972).

L'acide γ-aminobutyrique (ou **GABA**) est le principal neurotransmetteur inhibiteur du système nerveux central. Sa liaison sur les récepteurs GABA de type A ($GABA_A$) entraîne un influx d'ions chlorure dans le neurone permettant une hyperpolarisation du neurone post-synaptique, et ainsi une inhibition de son activité électrique. Réparties de manière quasiment ubiquitaire dans le cerveau, les synapses GABAergiques ont montré un rôle important dans la régulation de l'anxiété. Ce rôle est d'ailleurs utilisé par la classe de médicaments des **benzodiazépines** (BZDs), qui s'est imposée comme traitement clinique des troubles anxieux depuis la seconde moitié du $XX^{ème}$ siècle. En effet, ces molécules ont pour cible thérapeutique les un site de régulation situé sur le récepteur $GABA_A$. La liaison des BZDs sur ce site de régulation leur permet d'entrainer une meilleure fixation du neurotransmetteur sur son récepteur et de médier les différents effets cliniques associés aux BZDs : sédation, anxiolyse, myorelaxation et effet anticonvulsivant. Des sous-types de récepteurs $GABA_A$ ont depuis pu être identifiés et permettent d'expliquer les différents effets des BZDs (par exemple, le sous-type α_1 est associé aux effets sédatifs des BZDs tandis que le sous-type α_2 permet la médiation

de leurs effets anxiolytiques). De manière intéressante, il a aussi été montré que ces différents sous-types de récepteurs $GABA_A$ ne présentent pas la même répartition au sein des aires cérébrales. Cependant, malgré une efficacité reconnue et une diversité de traitements proposés (jusqu'à 11 benzodiazépines anxiolytiques commercialisées en France : Xanax®, Lexomil®, Temesta®, Valium®…), les BZDs cumulent différents effets secondaires handicapants : sédation, somnolence, amnésie… Cela n'exclut cependant pas les benzodiazépines d'être l'une des familles de médicaments les plus consommées en France – au moins 7 millions de Français ont consommé une benzodiazépine anxiolytique en 2012 (ANSM, 2013) – cette surconsommation étant principalement due à des erreurs de diagnostics de la part des médecins généralistes (Pélissolo *et al*, 2007). La nécessité de trouver des alternatives possédant moins d'effets secondaires fait donc partie des priorités de la recherche sur l'anxiété (Griebel and Holmes, 2013).

Ainsi, il a été montré qu'un **hydrolysat trypsique** de caséine α_{s1} bovine possède des propriétés anxiolytiques après injection intrapéritonéale (*i.p.*) dans un modèle d'anxiété chez le rat (Miclo *et al*, 2001). L'équivalent industriel (Lactium®, Ingrédia SA) présente les mêmes propriétés après administration orale chez le rat (Violle *et al*, 2006) ainsi que chez l'Homme (Kim *et al*, 2007; Messaoudi *et al*, 2005). Les propriétés anxiolytiques de cet hydrolysat ont depuis été confirmées chez différentes espèces : chat (Beata *et al*, 2007a), chien (Beata *et al*, 2007b), poney (McDonnell *et al*, 2013) et cheval (McDonnell *et al*, 2014). L'addition de propriétés anti-convulsivantes (Miclo *et al*, 2001) et hypnotiques (Guesdon *et al*, 2006; Dela Peña *et al*, 2016) chez le rongeur, un criblage du peptide actif sur le site BDZ du récepteur $GABA_A$ (Miclo *et al*, 2001) ainsi qu'une action *in vitro* sur l'activité des récepteurs $GABA_A$, cibles thérapeutiques des benzodiazépines (Dela Peña *et al*, 2016), ont permis de rapprocher cet hydrolysat de la famille des benzodiazépines. Cependant, contrairement à ces dernières, l'hydrolysat ne possède aucun des effets secondaires traditionnellement associés à cette famille de médicaments : tolérance, dépendance, sédation, effet amnésiant ou toxicité (Messaoudi *et al*, 2009; Dela Peña *et al*, 2016).

La recherche d'un peptide porteur de l'activité anxiolytique dans l'hydrolysat a finalement abouti à l'identification de l'**α-casozépine** (α-CZP), décapeptide de composition en résidus d'acides aminés identifiée : YLGYLEQLLR[6] (Miclo *et al*, 2001). Ce peptide possède, une

[6] Y : tyrosine, L : leucine, G : glycine, E : acide glutamique, Q : glutamine, R : arginine

affinité pour le site BDZ des récepteurs $GABA_A$ (bien que cette affinité soit 10.000 fois inférieure à celle du diazépam) et possède aussi des propriétés anxiolytiques chez le rat (Miclo *et al*, 2001). L'étude de la digestibilité *in vitro* de ce peptide par différentes enzymes gastriques et pancréatiques a permis de montrer que deux peptides étaient majoritairement formés, YLGYL* et YLGYLEQ* (Balandras *et al*, 2008, 2009), ce dernier possédant des propriétés anxiolytiques chez le rat et étant absorbé *in vitro* à travers un modèle de barrière intestinale (Cakir-Kiefer *et al*, 2011a, 2011b). Le mode d'action précis permettant la médiation des effets anxiolytiques de l'α-CZP reste encore inconnu, bien que de nombreux travaux pointent une action centrale du peptide.

Si l'anxiété a joué un rôle indispensable lors de l'évolution des espèces en préparant l'organisme à une situation dangereuse, une anxiété trop importante ou récurrente peut devenir néfaste pour l'organisme. En effet, depuis le début du XX^{ème} siècle avec les travaux de Sigmund Freud, différents **troubles anxieux** ont pu être identifiés chez l'Homme et répertoriés dans le Manuel diagnostique et statistique des troubles mentaux (American Psychiatric Association, 2013) : anxiété sociale, troubles paniques, phobies spécifiques, anxiété généralisée… Ces troubles sont devenus les plus fréquents des troubles mentaux dans les pays occidentaux, avec une prévalence de vie-entière touchant 21,6% de la population en France en 2007 (Haute Autorité de Santé, 2007). De nombreux travaux scientifiques, à la fois chez l'Homme et chez l'animal, ont permis de mieux comprendre les mécanismes centraux permettant la régulation de ce phénomène. Un ensemble de régions, regroupées anatomiquement sous le nom de **système limbique**, a ainsi pu être identifié et serait à l'origine de la régulation de l'anxiété, et plus généralement des émotions. Nous nous proposerons par la suite d'expliquer les phénomènes centraux impliqués dans l'anxiété au regard du modèle proposé par Calhoon et Tye, impliquant trois grandes étapes : l'interprétation de la menace, l'évaluation de celle-ci et enfin l'initiation de la réponse comportementale et physiologique (Calhoon and Tye, 2015). L'interprétation émotionnelle se fait principalement par le biais de l'amygdale et de la formation hippocampale, structures impliquées, entre autre dans la mémoire émotionnelle. L'évaluation de la menace se fera par des structures impliquées dans des processus cognitifs plus complexes, tel que le cortex préfrontal ou des structures impliquées dans les comportements motivés, tel que le noyau accumbens. Enfin, le déclenchement des réponses physiologiques se fera principalement par le biais de l'hypothalamus, à l'origine de l'axe hypothalamo-hypophyso-surrénalien qui déclenchera la libération des hormones du stress dans la circulation sanguine, tandis que les réponses comportementales seront principalement

pilotées par des régions du tronc cérébral. Il est à noter que les connections entre ces régions ne se font pas de manières linéaires et en sens unique, mais que les informations sont échangées entre toutes les régions sans sens particulier.

Pour permettre une meilleure compréhension du mode d'action de l'α-CZP, cette thèse appliquera différentes techniques expérimentales qui ont permis par le passé de mieux comprendre le fonctionnement central des troubles anxieux dans des modèles rongeurs. Un volet comportemental fera usage de différents **modèles comportementaux** développés aux cours de la seconde moitié du XXème siècle comme la boîte claire/obscure, l'open-field ou l'enfouissement défensif conditionné (Griebel and Holmes, 2013). Ces modèles permettent de reproduire des situations anxiogènes pour l'animal (forte lumière, grand espace inconnu ou choc nocif) dans un environnement contrôlé et reproductible. Un second volet permettra d'étudier l'influence de peptides anxiolytiques sur l'**activité neuronale** dans différentes régions cérébrales via l'évaluation de l'expression de la protéine c-Fos. Cette protéine est un facteur de transcription qui joue aussi le rôle de marqueur de l'activité neuronale suite à un stimulus précis. Sa révélation pour évaluer son expression peut, entre autres, se faire par immunofluorescence.

1.2 Travaux personnels

Bien que de nombreux résultats laissent à penser que le mode d'action de l'α-CZP est proche de celui des BZDs, certaines questions restent encore en suspens et des stratégies de recherche ont été envisagées pour y répondre :

- Plusieurs résultats laissent penser que les propriétés anxiolytiques de l'hydrolysat sont plus facilement mises en évidence chez les **individus particulièrement anxieux** (Briand, 2007; Guesdon *et al*, 2006; Kim *et al*, 2007; Palestrini *et al*, 2010). Il serait donc intéressant de comparer l'efficacité du peptide chez des individus anxieux et des individus non anxieux.
- L'hypothèse d'un mode d'action central est aujourd'hui privilégiée. L'impact de l'administration d'α-CZP sur l'**activité cérébrale** pourrait donc donner de premières informations quant à cette hypothèse, en mettant en évidence les effets du peptide sur l'activité neuronale dans des régions impliquées dans la régulation de l'anxiété et de comparer ces résultats avec une BZD de référence : le diazépam.

- Enfin, l'étude de **dérivés de l'α-CZP** pourrait nous permettre de comprendre si ces derniers agissent de la même manière que l'α-CZP ou si des différences peuvent être soulignées.

1.2.1 Sélection des animaux anxieux

Pour répondre à la première piste soulevée précédemment, deux populations d'individus doivent pouvoir être discriminées par leur niveau d'anxiété. Pour ce faire, les souris sont placées dans un dispositif permettant d'évaluer l'anxiété chez le rongeur : l'open-field. Ce dispositif consiste en une boîte carrée de 40 × 40 cm dont l'animal ne peut s'échapper. Le sol est délimité en 2 surfaces : le centre, particulièrement anxiogène pour les rongeurs, et la périphérie, moins anxiogène.

En mesurant les transitions des animaux d'une zone à l'autre pendant 5 minutes, il est ainsi possible d'évaluer l'anxiété des différents animaux, le rapport « *transitions vers le centre/transitions vers la périphérie* » étant un bon indicateur défini par la littérature (Bourin *et al*, 2007). Un total de 182 animaux, réparti en 5 lots différents a été ainsi testé dans ce dispositif. Comme aucune différence statistiquement significative n'a été observée entre les différents lots d'animaux, les résultats ont ainsi été regroupés. En regardant la répartition des animaux en fonction du paramètre cité ci-dessus, ceux-ci sont groupés autour de la moyenne et suivent une loi normale. Il n'existe ainsi pas de répartition bimodale de la population étudiée et il est donc impossible de discriminer les individus selon leur niveau d'anxiété.

En résumé : La difficulté de la discrimination de deux groupes distincts en fonction de leur niveau d'anxiété à l'aide du protocole utilisé ici impose donc l'utilisation d'un autre protocole pour différencier les individus en fonction de leur niveau d'anxiété. En effet, s'il est impossible de discriminer les individus en amont, il est cependant possible de créer une situation anxiogène pour une partie des individus, l'autre partie restant dans une situation connue et donc non anxiogène (situation contrôle).

1.2.2 Modulation de l'activité cérébrale par l'α-casozépine chez la souris

Un protocole de mise en situation anxiogène a été proposé : au sein de chaque groupe traité par voie intrapéritonéale (véhicule, α-CZP ou diazépam, une benzodiazépine anxiolytique de référence), les animaux sont répartis équitablement dans deux situations. La première est une situation contrôle où les animaux retournent dans leur pièce d'hébergement une fois l'injection réalisée, ce qui permet d'éviter des stimuli inconnus, anxiogènes pour les animaux ; la seconde est une situation anxiogène où les animaux sont placés dans le dispositif de la boîte claire/obscure. Ce dispositif est constitué de deux compartiments continus entre lesquels l'animal peut circuler librement. L'un des compartiments est éclairé, ce qui est anxiogène pour les animaux. Le temps passé dans ce compartiment est donc révélateur du niveau d'anxiété des animaux : plus celui-ci est élevé, moins les animaux sont anxieux. Les propriétés anxiolytiques de l'α-CZP ont ainsi pu être d'abord confirmées dans ce dispositif en comparaison avec le diazépam et sont en accord avec les résultats comportementaux obtenus précédemment chez d'autres espèces.

L'évaluation de la modulation de l'activité cérébrale des animaux a été effectuée par l'étude de la protéine c-Fos, mise en évidence par immunofluorescence. c-Fos est un facteur de transcription utilisé comme marqueur de l'activité neuronale (Herrera and Robertson, 1996; Kovács, 1998). En effet, son expression est très faible voire inexistante en conditions basales et un stimulus est nécessaire pour pouvoir observer la présence de la protéine dans le noyau de neurones activés. Les deux situations (contrôle et anxiogène) couplées aux différents traitements jouent le rôle de stimulus dans le cas présent. Un comptage automatique a été mis en place avec le logiciel ImageJ pour permettre une analyse plus objective des coupes obtenues.

Les cerveaux ont ainsi été prélevés après perfusion *in situ* des animaux au formol. Après congélation, des coupes sagittales, permettant l'étude simultanée de plusieurs régions sur une même coupe de cerveau, ont été réalisées au moyen d'un cryostat sur l'ensemble de l'hémisphère gauche. La densité des neurones c-Fos positifs dans différentes régions impliquées dans la régulation de l'anxiété permet de donner une évaluation de l'activité d'une région et de comparer les différentes situations et traitements entre eux.

En comparant les profils d'expression de c-Fos entre les deux situations pour les animaux ayant reçu une injection *i.p.* du véhicule, le stimulus créé par la situation anxieuse (boîte claire

obscure) a augmenté significativement le nombre de neurones exprimant c-Fos dans les régions suivantes : cortex préfrontal (×3,6), formation hippocampale (×5,4) et noyau accumbens (×2,1) entre autres. Concernant l'hypothalamus, la situation anxiogène n'augmente pas l'activité neuronale de manière globale mais spécifiquement dans les noyaux dorsomédian (×3,8), ventromédian (×5,2) et paraventriculaire (×4,4). Le stimulus anxiogène n'a, en revanche, aucun impact sur l'activité neuronale de l'amygdale.

Concernant la situation contrôle (retour à la pièce d'hébergement après l'injection), l'injection *i.p.* d'α-CZP ne modifie pas l'activité neuronale dans les régions étudiées en comparaison avec le témoin négatif (injection *i.p.* du véhicule). L'injection de diazépam diminue statistiquement en revanche le nombre de neurones exprimant c-Fos dans le noyau accumbens (×0,4) et le noyau paraventriculaire de l'hypothalamus (×0,3).

Enfin, si l'on considère la situation anxiogène (boîte claire obscure), l'injection *i.p.* d'α-CZP diminue de manière statistique le nombre de neurones c-Fos positifs au niveau de la formation hippocampale (×0,6), du noyau accumbens (×0,5) et des noyaux dorsomédian (×0,3), ventromédian (×0,3) et paraventriculaire (×0,6) de l'hypothalamus par rapport au véhicule. A l'inverse, cette même injection d'α-CZP augmente fortement le nombre de neurones c-Fos positifs dans l'amygdale (×2,8). Concernant l'injection de diazépam, celle-ci diminue statistiquement le nombre de neurones exprimant c-Fos dans le cortex préfrontal (×0,5), la formation hippocampale (×0,3), le noyau accumbens (×0,2) et les noyaux dorsomédian (×0,4), ventromédian (×0,4) et paraventriculaire (×0,6) de l'hypothalamus en comparaison avec le véhicule.

Les résultats obtenus avec le diazépam sont en accord avec les résultats obtenus par des études précédentes (Lkhagvasuren *et al*, 2014; de Medeiros *et al*, 2005). Les différences entre les études peuvent être expliquées par les différentes doses de diazépam utilisées ainsi que les différents tests utilisés pour servir de stimulus anxiogène. Ces résultats sont expliqués avec la répartition des récepteurs $GABA_A$/BZD dans les régions cérébrales : en effet, ceux-ci sont particulièrement concentrés au niveau du cortex préfrontal, de la formation hippocampale, de l'hypothalamus et du noyau accumbens (Richards and Möhler, 1984).

Concernant l'α-CZP, les résultats obtenus apportent un argument neurobiologique aux résultats comportementaux obtenus précédemment : en effet, l'absence d'effet du peptide sur

des populations peu anxieuses (Guesdon *et al*, 2006; Kim *et al*, 2007; Palestrini *et al*, 2010) peut être expliquée par l'absence d'effet sur l'activité neuronale dans une situation contrôle. En revanche, le peptide module l'activité neuronale dans une situation anxiogène, et ce de manière différente du diazépam, bien qu'il existe des similitudes entre les deux molécules. Ces différences pourraient s'expliquer par le fait que l'α-CZP possède une affinité spécifique pour un sous-type de récepteurs $GABA_A$, le peptide possédant des propriétés anxiolytiques mais pas d'effets secondaires traditionnellement associés aux BZDs (Messaoudi *et al*, 2009). Les récepteurs $GABA_A$ de type α_2 pourraient être de bons candidats pour l'affinité spécifique de l'α-CZP, car ils servent de médiateurs aux propriétés anxiolytiques des BZDs sans déclencher les effets secondaires, comme la sédation, qui reposent eux sur les sous-types α_1.

En résumé : Trois résultats majeurs peuvent être tirés de cette première étude. Il a ainsi été montré (i) qu'une situation anxiogène est nécessaire pour déclencher les effets anxiolytiques de l'α-CZP, (ii) qu'un peptide d'origine alimentaire injecté par voie *i.p.* peut moduler l'activité neuronale au niveau central, et (iii) que l'α-CZP n'agit pas sur l'activité cérébrale de la même manière que le diazépam, une BZD de référence.

1.2.3 Etude des dérivés de l'α-casozépine

Pour répondre à la question du mode d'action des peptides dérivés de l'α-CZP par rapport à celui de cette dernière, le pentapeptide YLGYL a été choisi après avoir vérifié ses propriétés anxiolytiques dans le modèle murin de la boîte claire/obscure. Le protocole présenté ci-dessus a donc été répété avec l'α-CZP et son dérivé YLGYL, mais cette fois uniquement dans la situation anxiogène. En effet, l'absence de stimulus anxiogène dans la situation contrôle présentée précédemment ne permettait pas de révéler les propriétés anxiolytiques de l'α-CZP ou du diazépam. Des coupes coronales, permettant une étude plus précises de certaines régions, notamment au niveau du tronc cérébral, ont été réalisées au niveau du cortex préfrontal, de l'amygdale et de trois structures du tronc cérébral : le noyau du faisceau solitaire, la substance grise périaqueducale et l'un des noyaux du raphé.

Les résultats obtenus précédemment avec les injections en *i.p.* d'α-CZP et de diazépam ont pu être confirmés avec les coupes coronales au niveau de l'amygdale et du cortex préfrontal (neurones c-Fos positifs ×2,4 pour l'α-CZP dans l'amygdale et ×0,3 pour le diazépam dans le cortex préfrontal) en utilisant un autre lot d'animaux. Les études supplémentaires réalisées dans

le tronc cérébral ont permis de montrer qu'en comparaison avec le véhicule, l'injection *i.p.* d'α-CZP augmente statistiquement le nombre de neurones c-Fos positifs du raphé (×2,0), tandis que celle de diazépam diminue de manière statistique les neurones c-Fos positifs de la substance grise périaqueducale (×0,5) et augmente ceux du noyau du faisceau solitaire (×2,4). Concernant YLGYL, l'injection *i.p.* du peptide augmente significativement uniquement le nombre de neurones exprimant c-Fos dans le raphé (×3,3).

Les régions cérébrales dont la modulation a été mise en évidence de manière globale ne sont pas homogènes et peuvent être subdivisées en différents noyaux qui possèdent des fonctions différentes. Les effets des différents traitements ne sont pas les mêmes au sein des différents noyaux. Ainsi, en évaluant les différents noyaux de l'amygdale séparément, il est observé que l'α-CZP augmente significativement l'activité des noyaux médian (×2,9) et cortical (×2,1), alors que YLGYL augmente uniquement l'activité du noyau cortical (×2,8) et le diazépam celle du noyau central (×2,8).

En résumé : YLGYL, un peptide dérivé de la digestion *in vitro* de l'α-CZP, présente lui aussi des propriétés anxiolytiques dans le modèle murin de la boîte claire-obscure. Il a été également montré que ce peptide module lui aussi l'activité neuronale de régions cérébrales impliquées dans la régulation de l'anxiété. Si les résultats concernant l'α-CZP ont pu être confirmés au niveau de l'amygdale et élargis à d'autres structures du tronc cérébral, comme un des noyaux du raphé, il semblerait qu'YLGYL n'exerce pas exactement les mêmes effets que l'α-CZP sur la modulation de l'activité neuronale, notamment au niveau de l'amygdale, où des différences au niveau de certains noyaux ont pu être observées, et du raphé.

1.3 Conclusion et perspectives

Cette thèse a donc montré que les propriétés anxiolytiques de l'α-CZP sont associées à une modification de l'activité cérébrale chez la souris dans différentes régions impliquées dans la régulation de l'anxiété. De plus, bien que présentant des similarités avec les effets du diazépam, une BZD de référence, sur la modulation de l'activité neuronale dans certaines régions (noyau accumbens, formation hippocampale, certains noyaux de l'hypothalamus), l'α-CZP possède certaines spécificités : une augmentation de l'activité neuronale dans l'amygdale et aucun effet sur le cortex préfrontal. Il en est de même pour YLGYL, peptide dérivé de l'α-CZP, qui ne

présente pas exactement le même profil de régulation de l'activité neuronale, que son peptide originel : activation spécifique de certains noyaux amygdaliens et augmentation significativement plus forte de l'activité d'un des noyaux du raphé par rapport à l'α-CZP. Enfin, il a été démontré qu'une situation anxiogène est nécessaire pour révéler cet effet central. Ces résultats constituent une avancée puisqu'à ce jour une seule étude, à notre connaissance, utilisant une β-casomorphine, avait montré la modulation de l'activité cérébrale par un peptide d'origine alimentaire même si dans notre cas le peptide a été administré par voie *i.p.* et non orale.

Si ces résultats auront permis de répondre à la question *« l'α-CZP exerce-t-elle une action sur le système nerveux central ?* », plusieurs questions ont pu à nouveau être soulevées pour avancer dans la compréhension du/des mode(s) et mécanisme(s) d'action de l'α-CZP.

Le site d'action du peptide est-il central ? En effet, s'il a été montré qu'une injection *i.p.* d'α-CZP module l'activité de certaines régions cérébrales, rien n'est encore prouvé sur la localisation de son site d'action. Si certains résultats laissent à penser que ce dernier est central (affinité pour le site de fixation des BZDs sur les récepteurs $GABA_A$), les réponses comportementales dans un modèle d'anxiété suite à l'injection intracérébroventriculaire de l'α-CZP permettraient de répondre à cette question. Il n'est cependant pas exclu que les effets anxiolytiques de l'α-CZP soit finalement dus à une ou plusieurs molécules intermédiaires générées suite à l'administration orale ou *i.p.* de l'α-CZP.

Que se passe-t-il dans le cas d'un complément alimentaire ? L'hypothèse d'une action centrale différente après une administration orale par rapport aux conditions déjà testées en injection *i.p.* pourrait être formulée. Les effets d'une administration orale de l'α-CZP, consommée comme complément alimentaire sur plusieurs jours, sur l'activité cérébrale pourraient apporter des résultats à comparer avec ceux obtenus après une unique injection *i.p.*

Quels neurotransmetteurs sont impliqués dans le mode d'action de l'α-CZP ? Après avoir identifié quelles régions cérébrales sont impliquées dans le mode d'action de l'α-CZP, la prochaine étape serait donc d'étudier quels neurotransmetteurs sont impliqués dans son mode d'action. Une vision plus précise du mode d'action pourrait être obtenue en injectant directement le peptide dans certaines régions cérébrales identifiées dans cette thèse (*e.g.* l'amygdale) *via* une chirurgie stéréotaxique. L'α-CZP ayant une affinité pour les récepteurs

$GABA_A$, il serait intéressant de déterminer si cette affinité est spécifique d'un sous-type de récepteur en utilisant des modèles knock-out et/ou des antagonistes spécifiques. Enfin, l'α-CZP présentant des similarités avec des peptides opioïdes dérivés de la caséine α_{s1}, l'affinité *in vitro* de l'α-CZP pour les différents récepteurs opiacés ainsi que l'implication de ces voies dans le mode d'action *in vivo* du peptide pourrait aussi être évaluées.

Comment étudier les effets centraux de l'α-CZP chez l'Homme ? L'α-CZP étant destinée à être consommée en tant que complément alimentaire par des individus anxieux, des études cliniques sont nécessaires pour obtenir des allégations santé pour le peptide. De plus, certaines informations obtenues chez l'animal ne peuvent pas toujours être extrapolées chez l'Homme. En effet, le système nerveux central n'étant pas développé de la même manière chez la souris et chez l'Homme, des différences existent dans la prise en charge des propriétés anxiolytiques de l'α-CZP par l'encéphale entre les deux espèces, notamment au niveau du cortex préfrontal. L'utilisation de techniques telles que l'encéphalographie dans un premier temps, ou l'IRM fonctionnelle dans un second temps, permettrait d'évaluer les effets de l'α-CZP dans une situation anxiogène (*e.g.* un stresseur de type psychologique comme le test de Stroop) chez l'Homme.

2 LIST OF COMMUNICATIONS

Publications

- **Benoit S**, Chaumontet C, Schwarz J, Cakir-Kiefer C, Tomé D, and Miclo L (2017). *Mapping in mice the brain regions involved in the anxiolytic-like properties of α-casozepine , a tryptic peptide derived from bovine α_{s1}-casein.* Journal of Functional Foods: 38, 464-473.

Oral communications and invited talks

- **Benoit S**, Chaumontet C, Schwarz J, Cakir-Kiefer C, Tomé D, and Miclo L. *Modulation of Cerebral Activity in Mice Induced by α-casozepine, a Anxiolytic Peptide Derived from Bovine αs1-Casein.* – 15th Naples Workshop on Bioactive Peptides. June 23-25, 2016. Naples, Italy.
- Invited talk at Vitafoods with Ingredia SA. May 11, 2016. Geneva, Switzerland.
- **Benoit S**, Chaumontet C, Schwarz J, Cakir-Kiefer C, Tomé D, and Miclo L. *Modulation of Cerebral Activity Induced by α-casozepine, a Benzodiazepine-like Peptide Derived from Bovine αs1-Casein.* – Experimental Biology. April 2-6, 2016. San Diego, USA.
- Tomé D, Soto M, Chaumontet C, **Benoit S**, Guillaumin M, Fromentin G, Andrey P, Burguet J, and Darcel N. *Spatial reorganization of Proopiomelanocortin (POMC)-expressing Neurons in the Arcuate Nucleus of POMC-EGFP Mice Resistant or Prone to Obesity.* – Experimental Biology. April 2-6, 2016. San Diego, USA.

Posters

- **Benoit S**, Chaumontet C, Schwarz J, Cakir-Kiefer C, Tomé D, and Miclo L. *Modulation of Cerebral Activity Induced by α-casozepine, a Benzodiazepine-like Peptide Derived from Bovine αs1-Casein.* – Experimental Biology. April 2-6, 2016. San Diego, USA.
- **Benoit S**, Chaumontet C, Schwarz J, Cakir-Kiefer C, Tomé D, and Miclo L. *Modulation de l'activité cérébrale chez la souris par l'α-casozepine, un peptide anxiolytique issu de la caséine αs1 bovine.* – Journées Francophones de Nutrition 2015. December 9-11, 2015. Marseille, France.

- **Benoit S**, Chaumontet C, Cakir-Kiefer C, Tomé D, and Miclo L. *Réponses comportementales et neuronales chez la souris suite à l'injection intrapéritonéale d'α-casozépine, peptide anxiolytique issu de la caséine αs1 bovine.* – Séminaire RP2E. January 15, 2015. Nancy, France.
- Darcel N, Soto M, Guillaumin M, Burguet J, **Benoit S**, Chaumontet C, Tomé D, Andrey P, and Fromentin G. *Représentation 3D des neurones POMC dans le noyau arqué chez les souris sensibles ou résistantes à l'obésité*. – Journées Francophones de Nutrition. December 10-12, 2014. Bruxelles, Belgium.
- Chalvon-Demersay T, Chaumontet C, **Benoit S**, Azzout-Marniche D, Fromentin G, Tomé D, and Even PC. *L'introduction d'un choix alimentaire diminue spécifiquement la prise de masse grasse des rats sensibles à l'obésité induite par un régime gras.* – Journées Francophones de Nutrition. December 10-12, 2014. Bruxelles, Belgium.

Award

- Graduate Student First Place of the Emerging Leaders in Nutrition Science Competition – Experimental Biology. April 2-6, 2016. San Diego, USA.

15th Naples Workshop on Bioactive Peptides 2016 – Abstract

Modulation of Cerebral Activity in Mice Induced by α-casozepine, a Anxiolytic Peptide Derived from Bovine α_{s1} Casein

Simon Benoit[1,2], Catherine Chaumontet[2], Céline Cakir-Kiefer[1], Jessica Schwarz[3], Daniel Tomé[2], Laurent Miclo[1]

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A tryptic hydrolysate derived from bovine milk α_{s1}-casein (CH) exerts an anxiolytic activity in different mammals including humans, after intraperitoneal (i.p.) or oral administration. Anticonvulsant effects and protective effects on sleep were also described in some species. Besides, compared to benzodiazepines (BDZ), the most commonly prescribed anxiolytic drug family, this CH wields no addiction, dependence, sedation or toxicity. It has since then been used as a dietary supplement in both human and veterinary medicine (claims were granted by the authorities in some countries).

The search for a bioactive carrier of the activity led to the discovery of a decapeptide, named α-casozepine (α-CZP), whose anxiolytic properties were confirmed in rats. This peptide also exhibits an affinity for the BDZ site of $GABA_A$ receptor, the molecular target of BDZ, but with an affinity 10.000 times lower than that of diazepam, a BDZ. Although a central action remains the main hypothesis of the mode of action of α-CZP, no regulation of the brain activity has been shown before. Thus, this study takes an interest in characterising the brain areas involved in the reduction of anxiety following i.p. injection of α-CZP by labelling neuronal activity in mice brain.

Male Swiss mice (8 per group) were placed in an anxiogenic situation (light/dark box) after an i.p. injection of either α-CZP (1 mg/kg), diazepam (1 mg/kg) or the vehicle used to solubilise the two molecules. One and a half hours later, mice were transcardially perfused with formalin and brains were picked up and cryosectioned. Cerebral activity was assessed in brain regions involved in anxiety regulation using c-Fos (a marker of neuronal activity) immunofluorescence. Results obtained for α-CZP and diazepam were compared to vehicle.

Anxiolytic effect of α-CZP on mice was demonstrated using the light/dark box. As far as cerebral activity is concerned, on the one hand, α-CZP reduced neuronal activity in both Nucleus Accumbens and Hippocampus, just as diazepam. α-CZP had no effect on Prefrontal Cortex activity, whereas diazepam decreased its activity. Besides, only α-CZP increased neuronal activity in the Amygdala, whereas diazepam had no effect in this region. No effect was observed for both α-CZP and diazepam in the Hypothalamus.

In conclusion, an intraperitoneal injection of α-CZP triggered a modulation of neuronal activity in brain regions implicated in anxiety regulation. The difference of modulation of neuronal activity between α-CZP and diazepam may explain the absence of side effects associated with CH.

Modulation of Cerebral Activity Induced by α-casozepine, an Anxiolytic Peptide Derived from Bovine α_{s1}-Casein

Simon Benoit[1,2,*], Catherine Chaumontet[2], Céline Cakir-Kiefer[1], Jessica Schwarz[3], Daniel Tomé[2], Laurent Miclo[1]

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BACKGROUND

α_{s1} bovine casein → Tryptic hydrolysate of α_{s1} bovine casein → Lactium® → α-casozepine YLGYLEQLLR

Substitute for benzodiazepines?

Anxiolytic activity: Humans, Rats, Cats, Dogs, Horses, Ponies

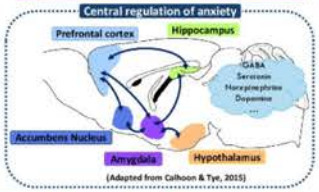
No side effects of α-CZP: no addiction, tolerance, sedation nor toxicity (Messaoudi et al., 2009)

α-casozepine / Diazepam

Low affinity for $GABA_A$ receptor (Miclo et al., 2001)

Hypothesis of an action on the CNS activation

Central regulation of anxiety

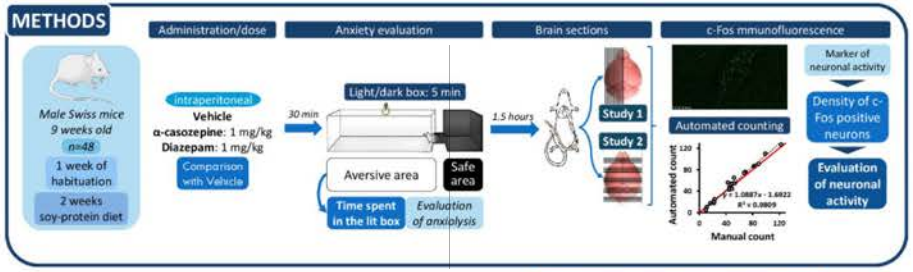


(Adapted from Calhoon & Tye, 2015)

What are the brain areas involved in the reduction of anxiety after administration of α-casozepine?

Evaluation of neuronal activity with c-Fos immunofluorescence (a marker of neuronal activity).

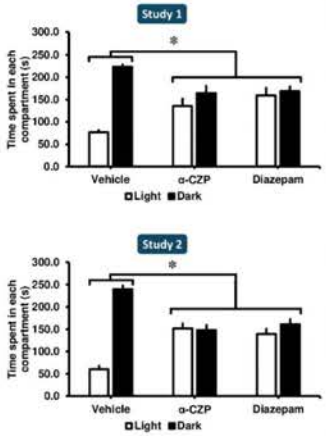
METHODS



RESULTS

Significant modulations of neuronal activity by α-CZP in several regions implicated in the regulation of anxiety (n=4/group)

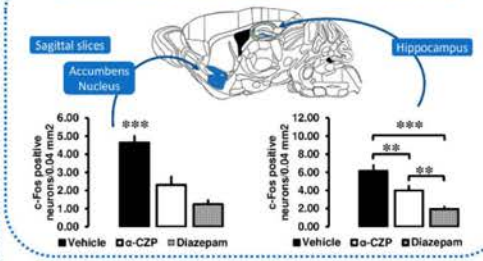
Anxiolytic effect of α-CZP in the light/dark box



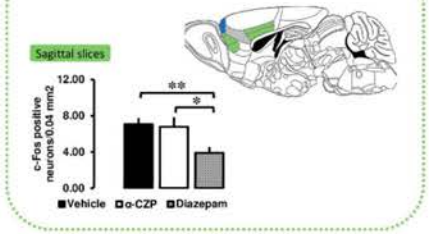
Both α-CZP and diazepam increase time spent in the lit box

n=8/group

α-CZP and diazepam decrease neuronal activity in Accumbens Nucleus (- 50%, - 75% respectively) and Hippocampus (- 35%, - 69% respectively)

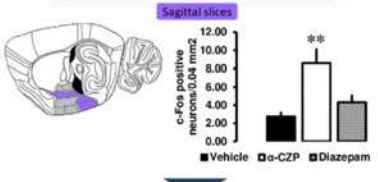


Only diazepam decreases neuronal activity in Prefrontal Cortex (-45%)

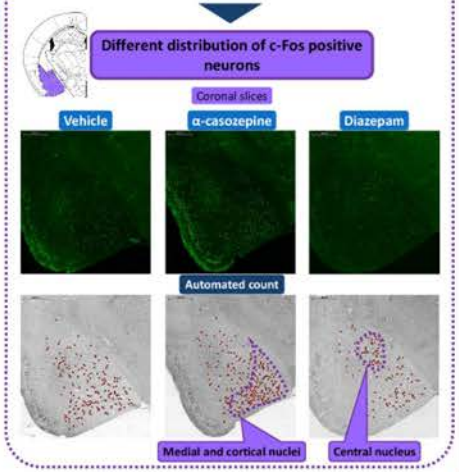


α-CZP and diazepam have not effect on the neuronal activity in the Hypothalamus

Only α-CZP increases neuronal activity in Amygdala (+216%)



Different distribution of c-Fos positive neurons



CONCLUSION & PERSPECTIVES

α-CZP increased time spent in the lit box

?

α-CZP decreased c-Fos positive neurons in Accumbens Nucleus and Hippocampus

?

α-CZP increased c-Fos positive neurons in Amygdala while Diazepam decreased c-Fos positive neurons in Prefrontal Cortex while

Different mechanism between α-CZP and Diazepam?

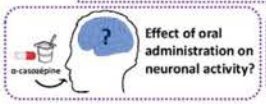
α-CZP has less affinity than diazepam for $GABA_A$ receptors (10,000 times) (Miclo et al., 2001)

$GABA_A$ antagonist blocks diazepam anxiolytic properties but not α-CZP's (Benoit et al., unpublished)

Is α-CZP a benzodiazepine-mimetic?

Flumazenil: antagonist of benzodiazepine receptors

Has α-casozepine affinity for other receptors (opiate, serotoninergic...)?



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Experimental Biology 2016 – Abstract

Modulation of Cerebral Activity Induced by α-casozepine, a Benzodiazepine-like Peptide Derived from Bovine αs1-Casein

Simon Benoit[1,2], Catherine Chaumontet[2], Céline Cakir-Kiefer[1], Daniel Tomé[2], Laurent Miclo[1]

[1] Équipe PB2P, UR AFPA, UC INRA 340, Université de Lorraine, Vandœuvre-lès-Nancy, France
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5061-ASN Mechanisms of Action and Molecular Targets of Dietary Bioactive Components

The tryptic hydrolysate of bovine αs1-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 GABAA 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 that of diazepam could also account for the absence of side effects observed with CH and this could be explained by the differences observed in the amygdala activation.

Modulations de l'activité cérébrale par l'α-casozépine, un peptide laitier aux propriétés anxiolytiques

Simon Benoit[1,2,*], Catherine Chaumontet[1], Céline Cakir-Kiefer[2], Daniel Tomé[1], Laurent Miclo[2]
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CONTEXTE

L' α-casozépine (α-CZP)

Issue de l'hydrolysat trypsique de la caséine α_{s1} bovine (fragment 91-100)

Y-L-G-Y-L-E-Q-L-L-R

Propriétés anxiolytiques (*in vivo*) (Miclo et al., 2001)

Une benzodiazépine mimétique ?

α-casozépine / Benzodiazépine — Similitudes de structure (Lecouvey et al., 1997)

In vitro — Affinité pour le récepteur des benzodiazépines (Miclo et al., 2001)

Les circuits centraux de l'anxiété

In vivo (Calhoon & Tye, 2015)

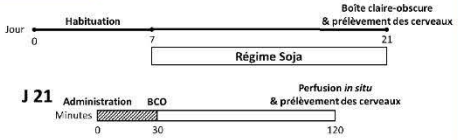
CeA, BLA : Amygdale
DR : Raphé dorsal
HPC : Hippocampe
Hyp : Hypothalamus
LC : Locus coeruleus
LS : Septum latéral
mPFC : Cortex Préfrontal
NAc : Noyaux accumbens
PAG : Aire périaqueductale
PB : Noyau parabrachial
Thal : Thalamus
VTA : Aire tegmentale ventrale

Les modifications comportementales observées avec l'α-casozépine lors d'un stress sont-elles intégrées au niveau cérébral ?
Mesure de l'activité neuronale par immunofluorescence anti c-Fos (un marqueur de l'activité neuronale).

Procédure expérimentale

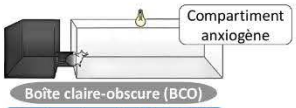
Souris Swiss ♂
9 semaines

Protocole expérimental



Administration *i.p.*

- Véhicule
- α-casozépine, 1 mg/kg
- Diazépam, 1 mg/kg

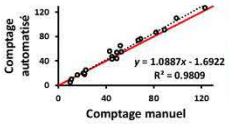


Evaluation de l'anxiété

Mesure de l'activité neuronale

1. Immunofluorescence

2. Mise au point d'un dénombrement automatisé

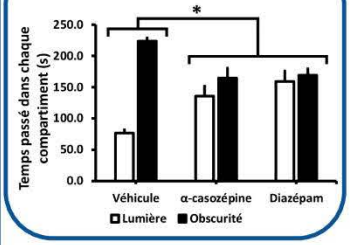


Densité de neurones c-Fos positifs → Evaluation de l'activité neuronale

3. Identification des régions cérébrales

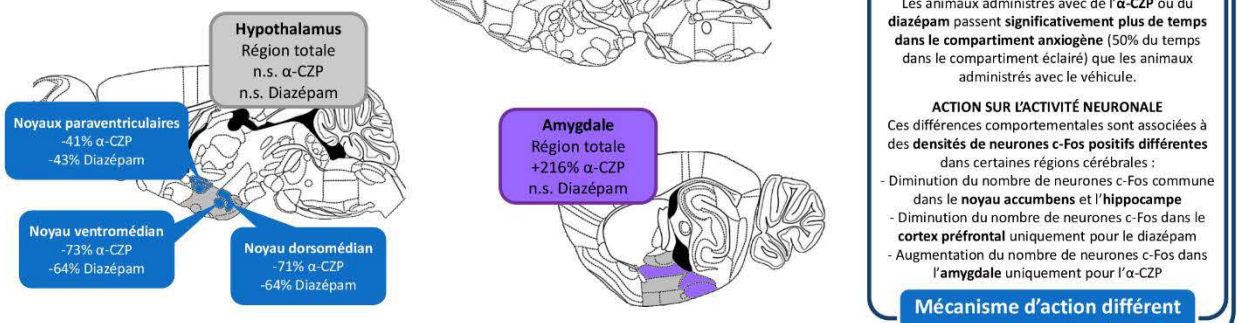
Résultats

Confirmation de l'effet anxiolytique



Modulations significatives de l'activité neuronale

- Aucune différence significative avec le véhicule
- ↓ significative de la densité de neurones c-Fos positifs pour α-CZP et diazépam
- ↓ significative de la densité de neurones c-Fos positifs uniquement pour le diazépam
- ↑ significative de la densité de neurones c-Fos positifs uniquement pour l'α-CZP



CONCLUSIONS

DIMINUTION DE L'ANXIÉTÉ

Les animaux administrés avec de l'**α-CZP** ou du **diazépam** passent **significativement plus de temps dans le compartiment anxiogène** (50% du temps dans le compartiment éclairé) que les animaux administrés avec le véhicule.

ACTION SUR L'ACTIVITÉ NEURONALE

Ces différences comportementales sont associées à des **densités de neurones c-Fos positifs différentes** dans certaines régions cérébrales :

- Diminution du nombre de neurones c-Fos commune dans le **noyau accumbens** et l'**hippocampe**
- Diminution du nombre de neurones c-Fos dans le **cortex préfrontal** uniquement pour le diazépam
- Augmentation du nombre de neurones c-Fos dans l'**amygdale** uniquement pour l'α-CZP

Mécanisme d'action différent entre α-CZP et diazépam ?

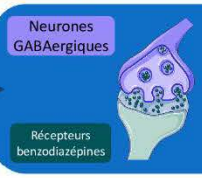
PERSPECTIVES

Y-L-G-Y-L-E-Q-L-L-R → Digestion → Y-L-G-Y-L → Y-L

Des peptides dérivés de l'α-casozépine possèdent-ils les mêmes propriétés fonctionnelles ?

(Kanegawa et al., 2010 ; Cakir-Kiefer et al., 2011)

Les effets centraux sont-ils retrouvés après une administration orale ?



Quels sont les mécanismes neuronaux mis en jeu ?

Système GABAergique

Journées Francophones de Nutrition 2015 – Abstract

Modulations de l'activité cérébrale par l'α-casozépine, un peptide laitier aux propriétés anxiolytiques

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

Comportement alimentaire – Fonction cérébrale

L'hydrolysat trypsique de la caséine αs1 de lait bovin (HTC) possède des propriétés anxiolytiques reconnues chez différentes espèces animales ainsi que chez l'Homme. De plus, contrairement aux benzodiazépines (BZD), classe de médicaments la plus prescrite en tant qu'anxiolytiques, l'HTC ne présente pas d'accoutumance, de dépendance, de sédation et de toxicité. L'hydrolysat est commercialisé sous la forme de complément alimentaire utilisé en médecine humaine et vétérinaire (allégations obtenues par l'AFSSA et la DGCCRF).

La recherche d'une molécule porteuse du mécanisme d'action a abouti à l'α-casozépine (α-CZP), un décapeptide dont les propriétés anxiolytiques ont pu être retrouvées chez le rongeur. Son affinité pour le récepteur GABAA a permis de rapprocher l'α-CZP des molécules de la famille des BZD, malgré une affinité plus faible que le diazépam, une BZD de référence.

Bien qu'une action centrale reste l'hypothèse principale du mécanisme d'action de l'α-CZP, aucune régulation de l'activité de zones cérébrales n'a été montrée auparavant. Ainsi, cette étude s'intéresse à caractériser chez la souris par un marquage de l'activité neuronale, les zones cérébrales impliquées dans la diminution de l'anxiété suite à une administration d'α-CZP.

Des souris Swiss sont habituées à un régime substituant les protéines laitières de l'alimentation par des protéines de soja, afin d'empêcher la formation de peptides laitiers endogènes. Les animaux (8 par groupe) sont placés dans une situation anxiogène (boîte claire-obscure) à la suite d'une injection intrapéritonéale d'α-CZP (1 mg/kg), de diazépam (1 mg/kg) ou du véhicule ayant servi à solubiliser ces molécules et perfusés au formol 1h30 après ce stimulus. Les profils de l'expression cérébrale de c-Fos (un marqueur de l'activité neuronale) sont révélés par immunofluorescence sur des coupes sagittales de cerveaux congelés.

Les effets anxiolytiques de l'α-CZP chez la souris ont été confirmés au moyen du dispositif de la boîte claire obscure. Concernant les études d'immunofluorescence, une diminution de l'expression de c-Fos au niveau du cortex préfrontal, de l'hippocampe, du noyau accumbens et de l'hypothalamus a été constatée après administration de l'α-CZP en comparaison avec le véhicule. Ces profils sont identiques à ceux suite à une injection de diazépam. Une augmentation de l'expression de c-Fos au niveau de l'amygdale, observée uniquement après administration d'α-CZP indique un mécanisme d'action différent de celui du diazépam.

L'administration d'α-CZP par voie intrapéritonéale permet une modulation de l'activité neuronale dans différentes régions impliquées dans la régulation de l'anxiété, ce qui permet en partie d'expliquer l'activité anxiolytique du peptide. Une liaison spécifique de l'α-CZP sur un sous-type de récepteur GABAA ($\alpha 2\beta 3\gamma 2$), dont la répartition coïncide avec les zones régulées par l'α-CZP et qui est le médiateur des effets anxiolytiques des BZD, pourrait être envisagée. Un mécanisme d'action différent de celui du diazépam pourrait être à l'origine de l'absence d'effet secondaire associée à l'HTC : l'activation d'une population de neurones GABAergiques dans cette région pourrait aussi être à l'origine de l'anxiolyse déclenchée par le peptide.

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Abstract

α-casozepine (α-CZP) is a decapeptide that mediates the anxiolytic-like properties of the tryptic hydrolysate of bovine α_{s1}-casein. Different properties of α-CZP leads to consider this peptide close to the benzodiazepine family, the most commonly used anxiolytic molecules. In contrast, other results suggest a distinct mode of action between α-CZP and benzodiazepines, especially the fact that the peptide does not have side effects. Although a central action remains the main hypothesis of the mode of action of α-CZP, no regulation of the brain activity has been shown before. The work achieved in this thesis displayed the fact that the anxiolytic-like properties of α-CZP, after a single intraperitoneal injection of the peptide, are associated with a modulation of cerebral activity in several regions linked to anxiety regulation in mice brains, such as the amygdala, the hippocampal formation, the accumbens nucleus and some nuclei of the hypothalamus or raphe. Besides, these modulations of neural activity are not exactly the same as those obtained after an injection of diazepam, a reference benzodiazepine, or YLGYL, a derivative of α-CZP, even though observed behaviours are similar. Eventually, it has been demonstrated that an anxiety-inducing situation is needed to trigger the central effects of α-CZP. This work allowed a better understanding of the mode of action of a bioactive peptide from alimentary origin that has a positive action on its consumer's mood and behaviour.

Keywords: *α-casozepine, bioactive peptide, anxiety, neural activity, animal behaviour*

Résumé

L'α-casozépine (α-CZP) est un décapeptide porteur des propriétés anxiolytiques de l'hydrolysat trypsique de caséine α_{s1} bovine. Différentes propriétés ont pu rapprocher ce peptide de la famille des benzodiazépines, les anxiolytiques les plus prescrits. Cependant, certaines différences, dont notamment une absence d'effets secondaires, permettent aussi de distinguer l'α-CZP des benzodiazépines. Bien que de nombreux éléments laissent penser qu'une action centrale reste l'hypothèse principale du mécanisme d'action de l'α-CZP, aucune régulation de l'activité de zones cérébrales n'avait été montrée jusqu'à présent. Ce travail de thèse aura donc pu montrer que les propriétés anxiolytiques de l'α-CZP sont associées à une modification de l'activité cérébrale chez la souris, après une unique injection intrapéritonéale, dans différentes régions impliquées dans la régulation de l'anxiété, notamment l'amygdale, la formation hippocampale, le noyau accumbens et certains noyaux de l'hypothalamus et du raphé. De plus, ces modifications de l'activité cérébrale ne sont pas exactement les mêmes que celles observées avec le diazépam, une benzodiazépine de référence, ni de celles obtenues avec YLGYL, un peptide dérivé de l'α-CZP, bien qu'il existe des similitudes dans le comportement de l'animal suite aux différents traitements effectués. Enfin, il a été démontré qu'une situation anxiogène est indispensable pour révéler cet effet central. L'ensemble de ce travail aura permis d'avancer dans la compréhension du mode d'action d'un peptide alimentaire ayant des effets positifs sur le comportement et les émotions de son consommateur.

Mots-clefs : *α-casozépine, peptide bioactif, anxiété, activité cérébrale, comportement animal*